

# Inversion of Proton Translocation in Bacteriorhodopsin Mutants D85N, D85T, and D85,96N

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**ABSTRACT** Proton translocation activity of bacteriorhodopsin mutants lacking the proton acceptor Asp-85 was investigated using the black lipid membrane technique. Mutants D85N, D85T, and D85,96N were constructed and homologously expressed in *Halobacterium salinarum* to yield a membrane fraction with a buoyant density of 1.18 g/cm<sup>3</sup>, i.e., identical to that of wild-type purple membrane. In all mutants, the absorbance maximum was red-shifted between 27 and 49 nm compared with wild type, and the pK<sub>a</sub> values of the respective Schiff bases were reduced to between 8.3 and 8.9 compared with the value of >13 in wild type. Therefore, a mixture of chromophores absorbing at 410 nm (deprotonated form) and around 600 nm (protonated form) exists at physiological pH. In continuous blue light, the deprotonated form generates stationary photocurrents. The currents are enhanced by a factor of up to 50 upon addition of azide in D85N and D85,96N mutants, whereas D85T shows no azide effect. The direction of these currents is the same as in wild type in yellow light. Yellow light alone is not sufficient to generate stationary currents in the mutants, but increasing yellow light intensity in the presence of blue light leads to an inversion of the current. Because all currents are carried by protons, this two-photon process demonstrates an inverted proton translocation by BR mutants.

## INTRODUCTION

The retinal protein bacteriorhodopsin functions as a light-driven proton pump in the plasma membrane of halobacteria. An overwhelming body of experimental data provides the basis for the beginning of a detailed understanding of the molecular events in the mechanism of proton translocation by BR. For recent reviews, see Mathies et al. (1991), Lanyi (1993), Rothschild (1992), Oesterhelt et al. (1992), and Krebs and Khorana (1993).

The proton-conducting channel, which is surrounded by the helices A, B, C, D, E, and G, is subdivided by the chromophore retinal into two half-channels: one of them connects the Schiff base with the extracellular medium, the EC channel, and the other links the Schiff base with the cytoplasm, the CP channel. Aspartic acids 85 and 96 (D85 and D96) were shown to play a central role in the proton translocation mechanism by analysis of proton currents in mutagenized BR molecules (Mogi et al., 1988; Butt et al., 1989; Marinetti et al., 1989). D85 in the extracellular part of the protein (EC channel) is deprotonated over the entire physiological pH range (pK at 3.2) and acts as an acceptor of the Schiff base proton. D96 is located in the cytoplasmic part of the protein (CP channel) and acts as the proton donor for the Schiff base (Holz et al., 1989; Otto et al., 1990; Gerwert et al., 1989; Miller and Oesterhelt, 1990). The lack of D96 slows the reprotonation of the Schiff base (decay of M intermediate) to s and renders the proton pump bulk pH-dependent. The

protonation of D85 or its removal prevents deprotonation of the Schiff base during the photocycle (formation of the M-intermediate) under standard conditions as exemplified by a D85E mutant with a pK of the glutamic acid higher than 7 (Subramaniam et al., 1990; Lanyi et al., 1992). Between these two important aspartic acid residues, retinal is switched by light between *cis* and *trans*, altering the accessibility of the Schiff base for protons from the cytoplasmic to the extracellular side, respectively. For a thorough discussion of this view of the vectorial transport, the reader is referred to Oesterhelt et al. (1992).

Transport measurements with mutants lacking the carboxylic group at position 85 revealed that no proton translocation activity could be detected upon illumination of the blue chromophore (Holz et al., 1989; Subramaniam et al., 1990; Lanyi et al., 1992) but could be restored if another proton acceptor like a glutamic acid or a histidine was introduced (Butt et al., 1989; Lanyi et al., 1992; Greenhalgh et al., 1992).

Interestingly, in a closely related anion pump, halorhodopsin, the amino acids equivalent to D85 and 96 are replaced by threonine and alanine. In a recent publication, we have shown that halorhodopsin can pump protons inwardly in a two photon process. This includes the light reaction of the HR<sub>410</sub> intermediate, which has a deprotonated Schiff base and a *cis* configuration of the retinal moiety (Bamberg et al., 1992).

These results created the obvious question of whether BR mutants lacking the proton acceptor D85, or both acceptor and donor (D96), could still function as proton pumps and whether the direction of translocation is changed depending on the isomerization state of the retinal before light absorption. Because of the lowered pK of the Schiff base in these molecules, a deprotonated all-*trans* form exists already in the dark, and proton translocation starting with this form and its

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Abbreviations used: Bacteriorhodopsin, BR; black lipid membrane, BLM; polymerase chain reaction, PCR; Fourier-transform infrared, FTIR.

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direction will be described in this report. On the other hand, if a deprotonated form can be established after a light reaction, this is expected to have 13-*cis* configuration, and the photoreaction of this 13-*cis* form is expected to result in a translocation direction, like in HR, opposite to the normal BR action. As in HR, in some mutants the rate of proton translocation can be increased by addition of azide, which was shown to accelerate de- and reprotonation reactions in HR (Hegemann et al., 1985) and the BR D96N mutant (Tittor et al., 1989). Therefore, the effect of azide will be of interest in this study as well.

## MATERIAL AND METHODS

### Construction of mutant strains D85N, D85T, D85,96N and isolation of mutated bacteriorhodopsins

Mutants were created in the shuttle-mutagenesis vector, pEF191 (Ferrando et al., 1993). It contains the origins of replication for *Halobacterium salinarum* and *Escherichia coli* and a resistance for mevinoline and, therefore, can be propagated and selected for in both organisms. The presence of the intergenic region from the phage F1 allows for the isolation of single-stranded vector DNA, enabling easy site-directed mutagenesis as well as sequencing.

Mutagenesis of pEF191 to obtain mutant D85T was carried out by the method of Kunkel et al. (1987). The transformation system (Cline et al., 1989) allowed the introduction of the mutated vector into the halobacterial strain L33 as described (Ni et al., 1990). Strain L33 contains a *bop* gene inactivated by stable insertion of the 520 bp ISH 2 element (DasSarma et al., 1983). Homologous recombination after transformation leads to two configurations, one creating the wild type and one the mutant phenotype. Because the mutants in position 85 are bluish, cloning allowed the phenotypic selection of the appropriate clones. In addition, the configuration regenerating the wild type is strongly disfavored versus the mutant and was not frequent (Ferrando et al., 1993). The mutation was verified by sequencing the chromosomal DNA of the selected clone as was the proper integration into the genome checked by PCR.

The mutation D85N was introduced by PCR and clones selected and analyzed as for mutant D85T. The double mutant D85,96N was prepared with the method of the gapped-duplex approach (Stanssens et al., 1989) and expressed in the strains L33 and HN5, which is halorhodopsin-negative (K. Rumpel, unpublished data). All three mutant strains were continuously passaged with phenotypic selection for high expression of BR and for independence of stability from mevinoline. This leads finally to a stable strain for D85,96N in HN5, but for the single mutations remaining, instability requires frequent recloning.

### Isolation of purple membranes

Isolation of purple membranes was according to the standard procedure (Oesterhelt and Stoekenius, 1974). All three strains produce bacteriorhodopsin partly in the form of purple membranes, i.e., membrane fractions of a buoyant density around 1.18 g/cm<sup>3</sup> that are isolated after water-induced lysis of the cells by sucrose density gradient centrifugation. The major portion of the mutated BRs, however, occurs in membrane fractions of lower buoyant density and was not used for our investigations.

### Retinal extraction

Membrane suspensions containing 5 nmol of the chromoprotein were mixed with an equal volume of isopropanol to denaturate the protein. Sodium phosphate buffer pH 7.0 was added to give a final concentration of 100 mM. Extraction of retinal was performed by addition of 1.5 volume of hexane

(2 times). The organic phase was dried from residual water by treatment with Na<sub>2</sub>SO<sub>4</sub>. The volume of the hexane was reduced to 100  $\mu$ l and injected into an HPLC system with a Lichrosorb 60 column and retinals eluted with 5% ethylacetate in hexane. All-*trans* and 13-*cis* retinal were identified by comparison with extractions from wild-type purple membranes, and 9-*cis* retinal was identified by co-chromatographed 9-*cis* standards. The yield of extraction was approximately 40%.

### Spectral measurements

Absorption spectra during acid-base titration were recorded on an Aminco DW2000 spectrophotometer (American Instrument Co., Silver Spring, MD) at a spectral bandwidth of 3.0 nm.

Flash photolysis experiments were carried out in a multichannel flash photolysis apparatus (Uhl et al., 1985). Bacteriorhodopsins were excited with a laser flash of 18-ns duration (excimer-dye laser system EMG100, FL3002, Lambda Physics, Göttingen, Germany).

### Electrical measurements

Black lipid membranes with an area of 10<sup>-2</sup> cm<sup>2</sup> were formed in a Teflon cell filled with the appropriate electrolyte solution (1.5 ml for each compartment). The membrane-forming solution contained 1.5% w/v diphytanoyllecithin (Avanti Biochemicals, Birmingham, AL) and 0.025% (w/v) octadecylamine (Riedel-de-Haen, Hannover, Germany) in *n*-decane to obtain a positively charged membrane surface (Dancshazy and Karvalhy, 1976). Membrane formation was controlled by eye, and the capacitance of each membrane was determined.

Purple membranes were suspended in distilled water (OD<sub>570</sub> = 5) and sonicated for 1 min in a sonication bath. Then aliquots of 30  $\mu$ l were added under stirring to the rear compartment of the Teflon cell containing the appropriate buffer. Photosensitivity of the system developed in time and reached a maximal and constant value after about 40 min.

The membrane was illuminated with light from a xenon lamp (100 W) and/or a mercury lamp (100 W). Light reached the membrane after passing through appropriate filters including a heat protection filter. The intensity of the continuous light source was up to 4 W/cm<sup>2</sup>. For "white" or "yellow" light cutoff filters,  $\lambda > 360$  or  $\lambda > 495$  nm (Schott, Mainz), respectively, were used. A K40 broadband interference filter (Balzers, Liechtenstein) served for "blue" light excitation. Light intensity was measured as described previously (Fendler et al., 1987).

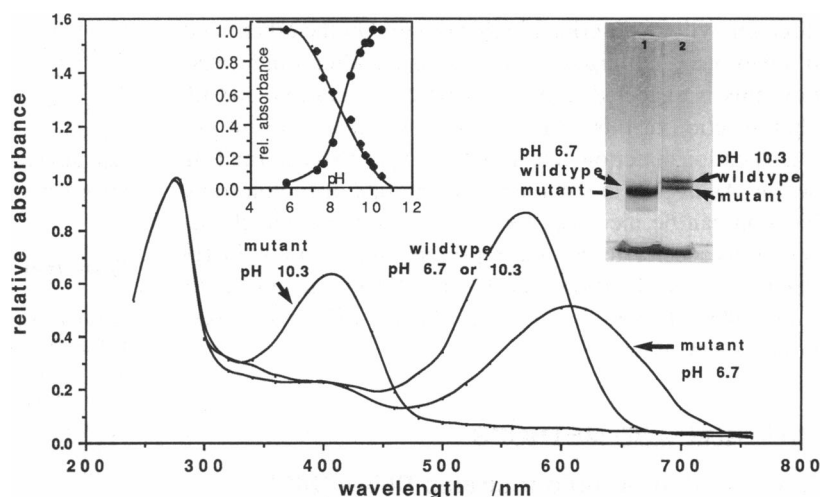
The suspensions on both sides of the black lipid membrane were connected to an external measuring circuit via Ag/AgCl electrodes, which were separated from the Teflon cell by salt bridges. The current was measured with a current amplifier (Keithley, model 427). Stationary currents were obtained after the addition of the protonophore 1799 (kind gift from Dr. P. Heydtler, DuPont Nemours), which permeabilizes the black lipid film for protons. Further details of the system were described earlier (Bamberg et al., 1979).

## RESULTS

### Spectroscopic properties of mutant BRs

Fig. 1 compares the mutant BR D85,96N membranes with wild-type membranes at pH 6.7 and 10.3, respectively. The mutant protein membranes are slightly denser at both pH values, and their spectra are quite different. At pH 6.7, the absorption maximum of the wild type is 568 nm but that of the mutant protein shifted to 608 nm. Only the mutant protein turns yellow upon alkalization, as found for deprotonated Schiff bases. The apparent pK of the transition from blue to yellow is 8.3 at 100 mM NaCl and decreases with increasing salt concentrations (not shown).

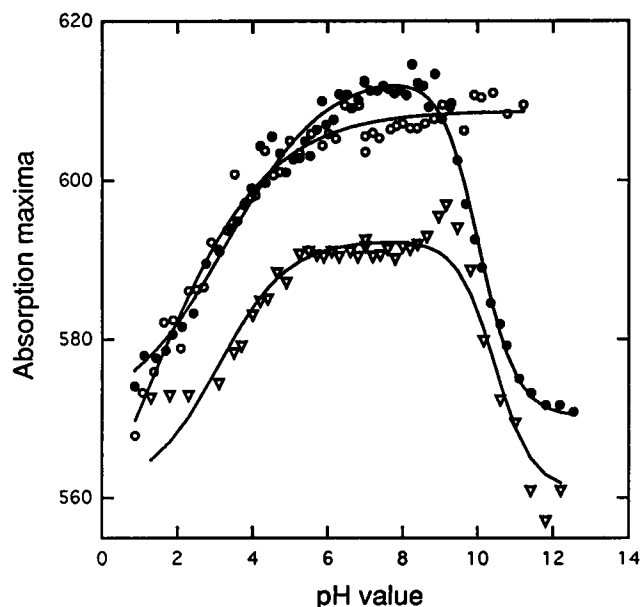
**FIGURE 1** Absorption spectrum and density of bacteriorhodopsin D85,96N. Spectra were taken at pH 6.7 (100 mM Tris/HEPES) and pH 10.5 (10 mM bicarbonate) and scaled for the same absorbance at 280 nm. On the inset upper right, the result of a sucrose density centrifugation is shown when the mutant membranes were mixed with wild-type at pH 6.7 and 10.5. The inset upper left shows the pH titration of the two species absorbing at 410 and 607 nm, respectively.



**TABLE 1** Absorption maxima and pKs values for different mutants of BR at position 85

	Absorbance maximum	pK of Schiff base
D85,96N	608	8.3
D85N	617	8.6
D85T	595	8.9
D85E	615(532 at pH 9.0)	>10
BR <sub>blue</sub>	605	not observable

Measurements were done in 10 mM Tris/HEPES, pH 6.7. pH titrations were carried out by adding small volumes of concentrated NaOH.



**FIGURE 2** pH dependence of the absorption maxima of mutants D85N (●), D85T (▽), and D85,96N (○). The solid lines are drawn to guide the eye.

The two single mutations D85N or T show qualitatively the same behavior. Their absorption maxima are listed together with the pK values of their Schiff bases in Table 1. An interesting shift of the absorption band of the protonated Schiff base is seen in these mutants. Unlike wild type, not a

**TABLE 2** Analysis of retinal isomers

	D85N	D85T	D85,96N
Dark-adapted			
All-trans	58	60	45
13-cis	40	37	55
9-cis	<2	3	<1
After illumination			
All-trans	51	49	44
13-cis	40	33	52
9-cis	7	18	4

Dark-adapted membranes were suspended in 10 mM Tris/HEPES, pH 6.7 and kept for 12 h at 4°C in the dark. Illumination was 10 min  $\lambda > 590$  nm (2.4 mW/cm<sup>2</sup>). Values are given in percent of total retinal.

red shift of the absorption band but a blue shift is seen at acidic pH values. Fig. 2 shows this pH dependence between values 2 and 12. This can be caused by the titration of a negatively charged group in the neighborhood of the cyclohexene ring of retinal and residue D115 would be a possible candidate. Indeed, molecular dynamics based pK calculations showed that the pKs of the Schiff base and D115 are closely correlated (Bashford and Gerwert, 1992). Similar calculations with mutated BR structures indicate that actually D115 displays a lowered pK when the charged residue at position 85 is removed (M. Nonella and D. Oesterhelt, unpublished data). Actually, solid state NMR experiments with 4-C<sup>13</sup> aspartate-labeled BRD85N revealed that a carboxylic group is titratable around pH 7.0 at a position that was attributed to asp-115 in wild-type (M. Eilers and M. Engelhard, personal communication). Further experimentation with more mutants will have to clarify this observation. Our experiments were carried out at pH 6.7, where the blue form is prevalent. At higher pH values, the absorbance maximum decreases again in mutants D85N and D85T, but not in mutant D85,96N, in addition to the occurrence of the deprotonated form absorbing maximally at 410 nm. This indicates again that other unidentified groups are interacting with the Schiff base in these mutants.

The photochemical properties of mutant D85N were described (Gergely and Varo, 1992), and the description was modified after additional FTIR measurements of the mutant

D212N showing similar spectroscopic behavior (Braiman et al., 1992). Similar to D85E (Lanyi et al., 1992) and other blue species that contain either a protonated amino acid or a noncarboxylic acid at position 85, D85T and D85,96N show the two essential features of this mutant BR group: 1) no M-intermediate is formed, but the L state converts directly into N, and 2) a photochemical side reaction of low quantum yield leads to 9-*cis* retinal in the binding site. Table 2 shows the isomeric distribution after photostationary illumination for mutant D85T, and only minor differences are found for the other two mutants described here. Different from wild type, light adaptation does not lead to complete conversion of retinal into the all-*trans* state. The photochemical activity of the 13-*cis* BRs is not considered here as interfering with the interpretation of our electric measurements described below because it was shown that in wild type the dark-adapted 13-*cis* form does not generate proton transport activity (Fahr and Bamberg, 1982). Actually, we have no hint that this should be altered by the mutations considered here. The 9-*cis* retinal state, on the other hand, is 1) mainly produced in red light not employed for ion transport experiments, and 2) is thermally stable on a long term basis, therefore not interfering with the all-*trans* photochemical reaction. Fig. 3 shows flash-induced difference spectra of the mutant D85N, and similar spectra were obtained for the other mutants. At pH 8.4, illumination with a series of laser flashes at 590 nm (20 s, 50 Hz repetition rate) results in the formation of a 410 nm absorbing species (Zimanyi et al., 1992) (Fig. 3,

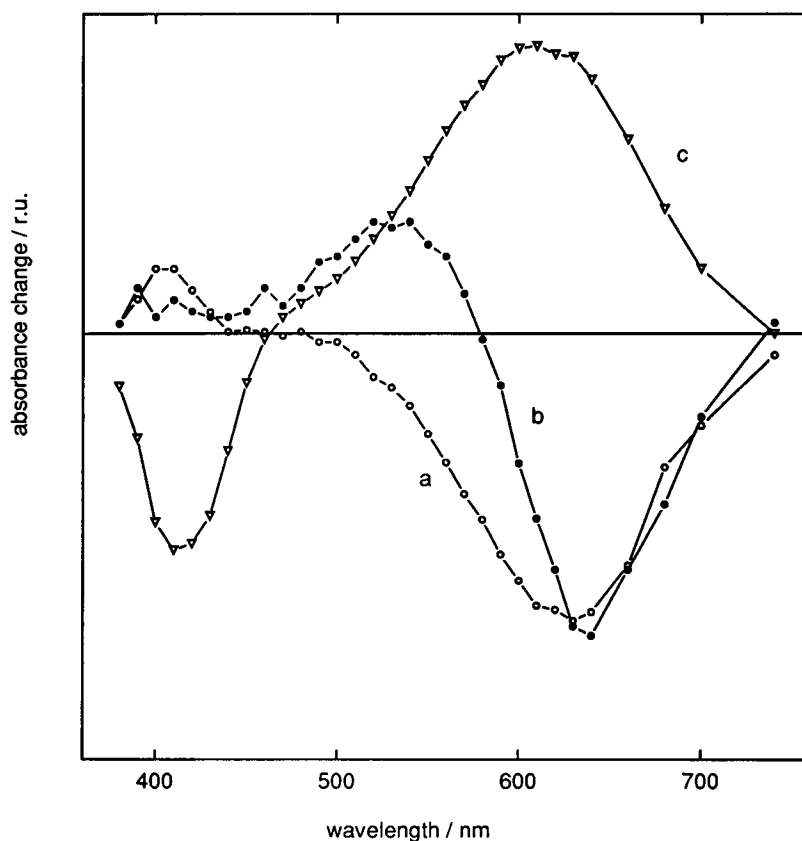
trace *a*, open circles), but the ratio of 1:4 of the maximum at 410 compared with the maximum at 640 clearly indicates that this is not the well known M-BR difference spectrum seen in wild type with a ratio of 2:3 upon flash illumination (data not shown). This indicates that other processes contribute to that spectrum like the above-mentioned formation of a 9-*cis*-containing photoproduct stable on the time scale of the flash experiment. At pH 7.0, the same laser flash produces an intermediate absorbing maximally at 540 nm (Fig. 3, trace *b*, closed circles), which is assigned as a N intermediate according to FTIR measurements (Braiman et al., 1992).

At alkaline pH 9.4, where the yellow-colored 410 nm-absorbing chromophore form of BR absorbs, repetitive (50 Hz) illumination with blue laser flashes causes the blue-colored 610 nm-absorbing form of the chromophore to rise to a steady-state level (Fig. 3, trace *c*, triangles). In the dark, the initial concentrations of the two forms are reestablished. Thus, either yellow or blue light initiates reversible photo-reactions at moderate alkaline pH, interconverting a deprotonated ( $\lambda_{\max}$  at 410 nm) and a protonated form ( $\lambda_{\max}$  at 610) of the chromoprotein.

### Electrical measurements

For the following, it is assumed that attachment of mutated membrane fragments is of the same orientation as for wild-type purple membranes. This seems reasonable, because only

FIGURE 3 Flash-induced difference spectra of BR D85N. 5  $\mu$ mol of a PM suspension in 10 mM Tris/HEPES, pH 8.5, were illuminated (a) 20 s by a 50 Hz, 590 nm Laser (quasi continuous illumination), (b) single laser flash (5 mJ, 18-ns duration), and (c) 20 s with 420 nm laser flashes with 50 Hz repetition rate. Traces shown in *a* and *c* are in the photostationary state ( $\sim 10$  s after onset of the actinic light); *b* is 650 ms after the flash.



internal amino acids were exchanged and attachment is mainly directed by surface charges.

We want to stress the point that all electrical measurements reported were performed at pH 6.7, where a significant amount of the molecules are in all-*trans* configuration (table 2).

In Fig. 4, capacitive photocurrents of membranes of mutated BRs attached to a lipid bilayer are shown. Illumination was with yellow (*top row*), white (*middle row*), and blue (*bottom row*) light. Obviously, the capacitive currents as the responses to "light on" and "light off" are of opposite sign in yellow and blue light, respectively, in all three mutated BRs.

Because of the time resolution of the setup, the capacitive currents cannot reflect the retinal isomerization steps (Fahr et al., 1981) but should be rather connected to proton uptake or release processes after light-excitation similar to what was shown for halorhodopsin (Bamberg et al., 1992). From the direction of these capacitive currents and from the fact that they are converted into stationary ones upon addition of protonophores (see below), it is concluded that illumination with blue light should then result in the uptake of a proton spectroscopically seen by conversion of the deprotonated 410 species to the protonated one absorbing at 610 nm. Therefore, this uptake should be from the same side as a proton was released upon illumination with yellow light, because the two capacitive currents have opposite signs.

Upon addition of the protonophore 1799, the underlying lipid membrane becomes selectively permeable to protons and the occurrence of stationary photocurrents now clearly indicates that protons carry the charge of the observed currents (Fig. 5). No stationary photocurrent can be observed in either mutant upon illumination with yellow light (*a, d, and g*), and for mutant D85N this finding was already observed

by others (Subramaniam et al., 1990; Miercke et al., 1991). But in blue light a stationary photocurrent is observable in all three mutants (traces *c, f, and i*) with somewhat different evolution in time and different amplitudes. This might be because of different kinetics, which will be the object of detailed studies in the future. The most surprising observation was after a change from blue light to white light illumination. The stationary currents changed their sign in all three mutants (traces *b, e, and h*). In addition, the size of the currents was comparable with that measured in blue light. Clearly, this depends on the light intensity, as shown in Fig. 6. Constant illumination with yellow (Fig. 6 A) or blue (Fig. 6 B) was used, and additional blue or yellow light was continuously increased in intensity. Only a neglectable photocurrent could be obtained with yellow light alone (blue light zero in Fig. 6 A), and an increasing positive current was obtained with increasing blue light intensity (Fig. 6 A). In contrast to this, in mere blue light a negative stationary photocurrent of  $-135$  nA decreases by increasing additional yellow light intensity, passes the zero line, and levels above 100 nA (Fig. 6 B). About the same levels are obtained in Fig. 6 A.

### Effect of azide

The stationary photocurrents demonstrated in Figs. 5 and 6 were obtained in the presence of 50 mM azide. In D85N and in D85,96N, stationary photocurrents are drastically reduced in the absence of azide, but are still present. Therefore, it is concluded that the reversal of the stationary currents is not caused by azide. In Fig. 7, stationary photocurrents of the mutant D85N are shown as an example in yellow, white, and blue light (traces *a, b, c*, respectively) in the absence of azide and after addition of 50 mM azide (*d, e, and f*). In yellow light, a small and transient negative current is not influenced

FIGURE 4 Capacitive photocurrents of BR D85,96N (*a-c*), D85N (*d-f*), and D85T (*g-i*) in 0.1 M NaCl and 20 mM Tris/HEPES, pH 6.7. The protein was illuminated by yellow ( $\lambda > 515$  nm,  $2$  W/cm<sup>2</sup>) light (traces *a, d, and g*), white ( $\lambda > 360$  nm,  $2.8$  W/cm<sup>2</sup>) light (*b, e, and h*) or blue ( $360 < \lambda < 420$  nm,  $0.7$  mW/cm<sup>2</sup>) light (*c, f, and i*). The vertical bars indicate the measured currents in nA; the horizontal bar indicates the time, which is the same scale in all experiments shown.

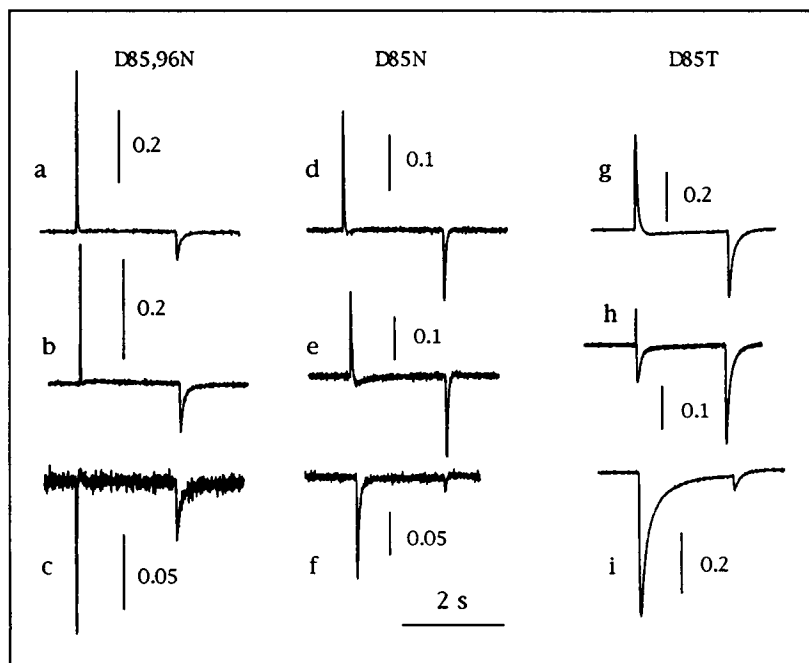


FIGURE 5 Examples of stationary photocurrents in yellow, white, and blue light. The conductance of the lipid bilayer was in the presence of  $16 \mu\text{M}$  1799 between 20 and 50 nS. Light conditions, indication of the traces, and scaling are the same as described in Fig. 4.

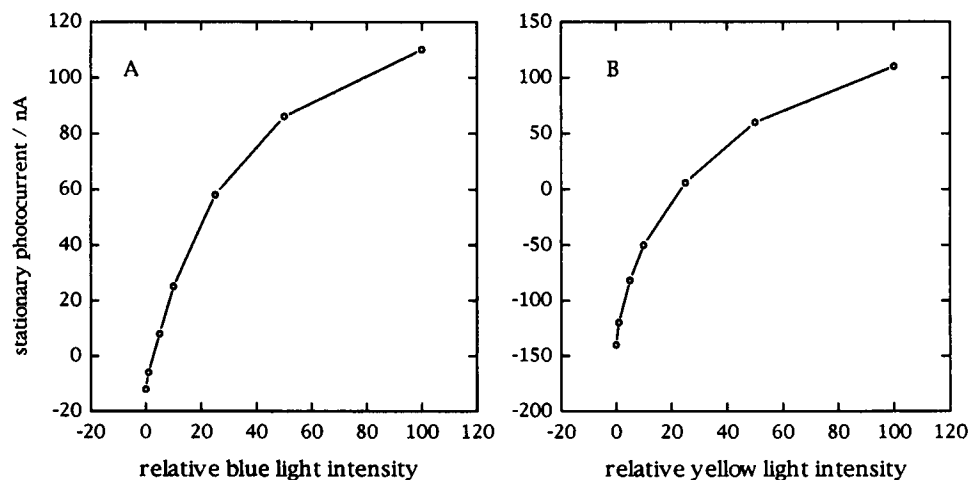
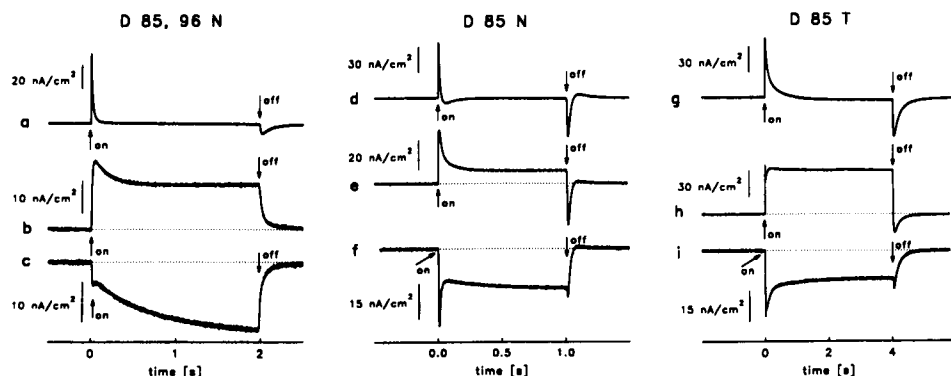


FIGURE 6 Titration of the stationary photocurrents with increasing blue (A) or yellow (B) light intensity in the presence of constant yellow (A) or blue (B) light. 100% light intensities refer to the intensities given in Fig. 4.

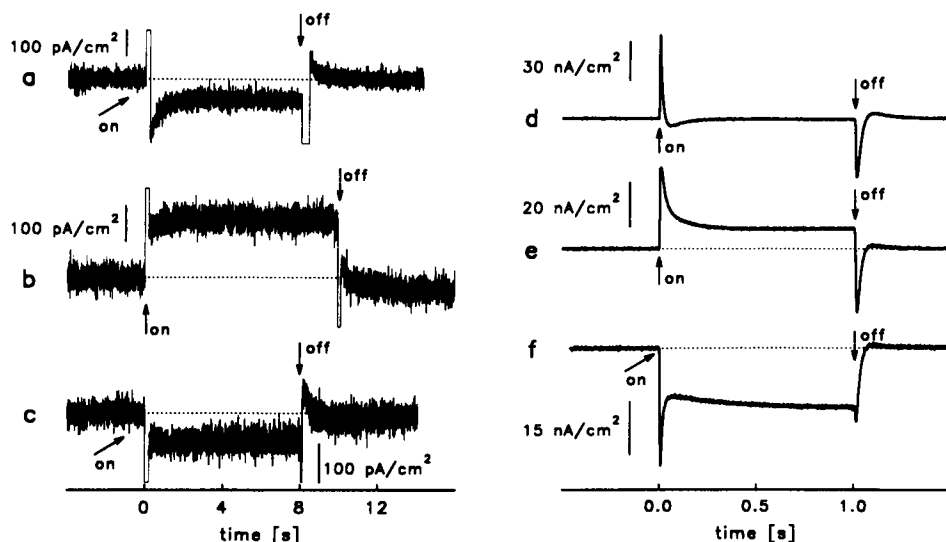


FIGURE 7 Effect of azide on stationary photocurrents. Photocurrents of BR D85N in the presence of  $16 \mu\text{M}$  1799 after (a-c) and before addition (e-f) of 20 mM azide. Light conditions were as described for Fig. 4.

by addition of azide. A drastic increase of the negative photostationary current by azide is observed in blue light (Fig. 6, c and f). In white light, a stationary current of opposite sign is observed but also drastically enhanced by azide. This finding clearly excludes that the inversion of the currents is caused by azide.

Mutant D85T showed no increased current after addition of azide under any condition of illumination. At the moment,

no explanation can be given why in D85T no azide effect is measurable, but it should be stated that, in general, effects are only expected if rate-limiting steps in the catalytic cycle are accelerated by azide. We started experiments to measure the time-resolved currents in these mutated BRs, and these experiments will show which step(s) are affected by azide. But it should be stated very clearly that the action of azide in mutant D85N or D85N,96N is an additional one to the one

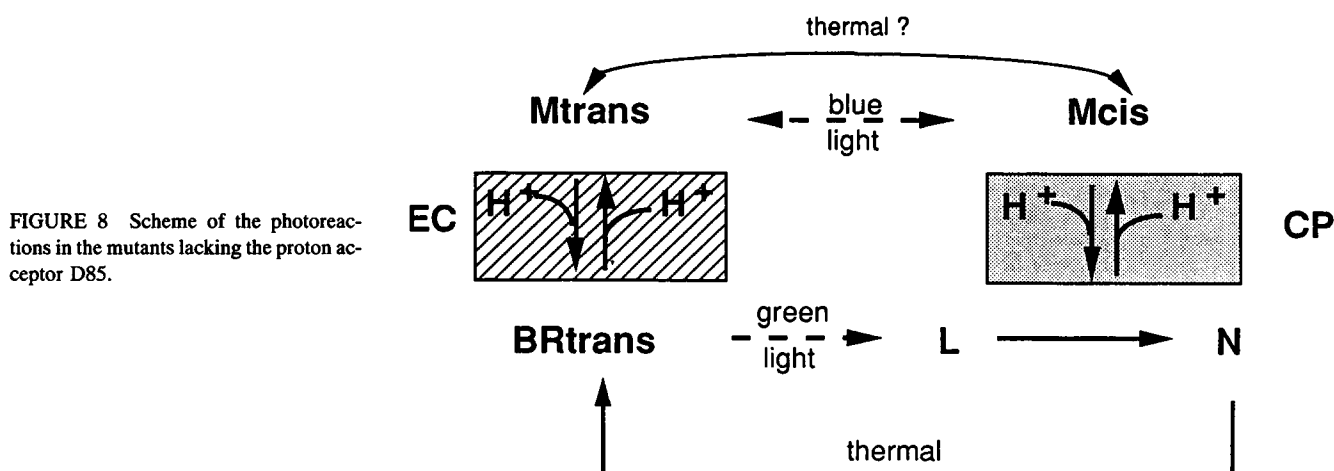


FIGURE 8 Scheme of the photoreactions in the mutants lacking the proton acceptor D85.

described for mutant D96N (Tittor et al., 1989). For a more detailed spectroscopic description of the azide effect in D85N, see Tittor et al. (1994).

## DISCUSSION

The attachment of wild-type purple membranes under the conditions of our experiments is with the extracellular surface facing the BLM. We expect the mutant purple membranes to orient for attachment the same way as wild-type membranes because only internal point mutations in the protein structure were made. In all likelihood, such changes should not alter surface charge densities, which are responsible for orientation of attachment.

Using the very sensitive BLM technique, it was then shown that bacteriorhodopsin mutants lacking the D85 can invert the translocation direction of protons under experimentally well defined conditions, i.e., two-photon excitation. Even if the assumption that attachment of the mutant membrane fragments is the same as for wild-type membranes were incorrect, the fact that the stationary currents are reversed remains valid. This can be stated because it is solely the ratio of blue and yellow light intensities that determines this inversion, and yellow light alone does not cause stationary currents. The only alternative explanation to a reversion in direction of proton translocation in blue and yellow light would be a yellow light-induced change in the sidedness of attachment. This would make the blue light-induced stationary current dependent on the conditions of preillumination, which is not observed experimentally.

The experiments in Figs. 4 and 5 demonstrate that the proton uptake in blue light and the release of protons in yellow light are through the same half-channel, and this was further corroborated by the action spectra of the respective capacitive currents of opposite sign. It cannot be derived immediately, however, if the half-channel concerned is CP or EC. Two observations favor the interpretation that it is CP. First, in wild-type BR a deprotonated Schiff base in 13-*cis* (M intermediate) upon absorption of a blue photon is converted to all-*trans* and picks up a proton through EC (Ormos et al., 1978). It seems unlikely that the opposite reaction, i.e.,

photoisomerization of the deprotonated Schiff base from *trans* to *cis* would cause the same directionality of proton uptake in the mutants.

The second observation was made during FTIR experiments. After excitation of BR mutants with yellow light, a deprotonation of D96 occurs without the Schiff base being deprotonated (Cao et al., 1993). This strongly argues that proton release in yellow light, and therefore proton uptake in blue light, is through the CP channel.

The possibility of addition of ion-selective ionophores to obtain stationary photocurrents is one of the main advantages of the BLM system as used. Therefore, beyond doubt all stationary currents shown are exclusively caused by proton movements because only the protonophore 1799 is required. The amount of purple membranes attached to BLM membrane varies from experiment to experiment within certain limits and, therefore, a quantitative comparison of data from different experiments is difficult. An estimation of the efficiency of the blue light-driven stationary currents in the mutants (around 20 nA/cm<sup>2</sup> in the presence of azide), however, can be obtained from the comparison with the value of 2–5 μA/cm<sup>2</sup> (in yellow) in wild type. Because of the lower intensity of the blue light compared with yellow light, a factor of four is already expected because both experiments were performed in the linear region of the intensity dependence. Moreover, the quantum yield of the blue light reaction is not known exactly for the all-*trans* form, but first experimental results indicate that it is a factor of three lower than the yellow light reaction in wild type (M. Wahl, personal communication). Therefore, the stationary currents measured in blue light should be similar to the ones measured for wild type under standard conditions.

The question then arises: How can the molecule achieve the different directions of proton translocation? The spectroscopic measurements may help to give the answer. All three mutants have in common that the pK of the Schiff base is drastically lowered to a value around 8.5 compared with the wild-type value of >13 (Druckmann et al., 1982). Therefore, a mixture of two chromophoric species, both in all-*trans* configuration, of the retinal exists around this pH value. Both

species were shown to undergo reversible photoreactions, and in both cases the first reaction should be a *trans-cis* isomerization. Excitation of the protonated 610 form results in predominant formation of L- and N-like intermediates and subsequent repopulation of the initial state 610 (Gergely and Varo, 1992) by protein-catalyzed reisomerization of the retinal. But upon sustained illumination at increased pH, formation of an intermediate absorbing maximally at 410 nm can be observed (Fig. 3 a). This situation is similar to halorhodopsin, where also a slow deprotonation reaction of the Schiff base can be observed as a side reaction during the photocycle. Clearly, the retinal configuration of such a light-induced 410 nm species should be 13-*cis*.<sup>1</sup> The amount of the produced *Mcis* is determined by the ratio of the kinetic constants describing the N decay and the formation of *Mcis*. If *Mcis* absorbs a blue photon and reisomerizes to the all-*trans* state, the uptake of a proton through EC in the all-*trans* configuration is the only step necessary to restore the initial state. This resembles the cyclic reaction, as was reported for halorhodopsin, resulting in an inwardly directed proton translocation in a two photon process (Bamberg et al., 1992).

On the other hand, illumination of *Mtrans* with a blue light will result in formation of *Mcis*. Obviously, the thermal decay after reprotonation of the Schiff base via the N intermediate to 610 nm species is the only way to complete the cycle. The fact that this process is strongly dependent on azide (increasing of the stationary photocurrents in the blue by a factor of 50) indicates that the decay of the *Mcis* might be the rate-limiting step in this reaction pathway. The release of the Schiff base proton from the 610 nm species to the outside is then the last reaction necessary to complete the cycle, which started from *Mtrans*. In Fig. 8, the reaction cycles leading to the respective protonic currents are summarized. No new assumptions beyond the known facts about partial reactions in BR and HR have to be made.

During the review process of this manuscript, one referee pointed out that thermal isomerization of the chromophore after deprotonation was reported for mutant D85N (Turner et al. 1993) after a pH change from 6.5 to 10.5. This would potentially lead to a shunt in the transport cycle shown in Fig. 8. However, thermal equilibration at pH 6.7 was not demonstrated and, if present, would not lead to proton transport activity because of the lack of a driving force. What remains is a potentially decreased transport activity caused by kinetic competition of thermal and photochemical steps. Upon light saturation, only the branching ratio of *Mcis* to *Mtrans* and *Mcis* to L/N would be determinant for efficiency and potentially influenced by azide.

The inversion of stationary photocurrents demonstrates that instead of consecutive pK alterations in a proton-conducting network in the protein being prerequisite for proton translocation, an active element within the protein de-

fines the vectoriality for the proton translocation. This active element is the Schiff base of retinal, and its accessibility for protons from the cytoplasmic channel or extracellular channel depends on its *cis* and *trans* states. The CP and the EC half-channels contribute to proton diffusion speed to and from the Schiff base, and D85 and D96 help drastically to optimize this process kinetically but are not required for vectorial catalysis. We cannot explain how retinal achieves the different accessibilities in the *cis* and the *trans* state, but obviously different geometries must play an important role. In addition, the Schiff base in the *cis* and *trans* state has a different pK, and this difference determines the thermodynamic potential of the mutated proton pumps.

The blue light-driven proton translocation is the first example where the temporal order of release and uptake of protons in BR is inverted. It directly demonstrates that the Schiff bases of the all-*trans* and 13-*cis* form have different affinities for the proton and different accessibilities. Although the 13-*cis* form accepts and releases protons preferentially through the CP channel, the *trans* form releases and accepts protons preferentially through the EC channel. The ratio of selectivities is not known but is expected to be high because in the absence of azide in mutant BR D96N, the 13-*cis* (M)-state can live for minutes without receiving a proton through EC. On the other hand, it is this channel through which in wild type the all-*trans* state exchanges protons with the bulk phase on the millisecond time range.

Moreover, it is shown that the vectoriality of transport is not determined by the aspartic acids 85 and 96. Thus, only the two half-channels and their relationship to the retinal *cis/trans* switch can constitute a vectorial catalyst.

In summary, we have shown that in mutants lacking the proton acceptor D85 and, in part, the proton donor D96:

- (i) stationary photocurrents in blue light can be measured, comparable with the ones measured in yellow light in wild-type,
- (ii) the vectoriality of the translocation in blue light is the same as in wild type; therefore, D85 and D96 do not define the vectoriality of proton translocation,
- (iii) inversion of the stationary currents in blue light by additional yellow light can be obtained,
- (iv) only protons are responsible for the observed blue light-driven stationary currents.

Even though many details are still missing for a description of molecular events of the individual steps in the catalytic cycle of bacteriorhodopsin and halorhodopsin, it becomes more and more clear that the key element for ion translocation is the light-triggered switch between all-*trans* and 13-*cis* or 13-*cis* to all-*trans*.

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<sup>1</sup> For a better discrimination we denote the light-induced, maximally at 410 nm absorbing species at *Mcis*. In comparison, we denote as *Mtrans* the one which is obtained from the all-*trans* chromophore absorbing maximally at 610 nm after deprotonation of the Schiff base.

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