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Aspartic acids 96 and 85 play a central role in the function of bacteriorhodopsin as a proton pump

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A spectroscopic and functional analysis of two point-mutated bacteriorhodopsins (BRs) from phototrophic negative halobacterial strains is reported. Bacteriorhodopsin from strain 384 contains a glutamic acid instead of an aspartic acid at position 85 and BR from strain 326 contains asparagine instead of aspartic acid at position 96. Compared to wild-type BR, the M formation in BR Asp^{85} → Glu is accelerated ~10-fold, whereas the M decay in BR Asp^{96} → Asn is slowed down ~50-fold at pH 6. Purple membrane sheets containing the mutated BRs were oriented and immobilized in polyacrylamide gels or adsorbed to planar lipid films. The measured kinetics of the photocurrents under various conditions agree with the observed photocycle kinetics. The ineffectivity of BR Asp^{85} → Glu resides in the dominance of an inactive species absorbing maximally at ~610 nm, while BR Asp^{96} → Asn is ineffective due to its slow photocycle. These experimental results suggest that aspartic acid 85 is involved in the proton release from BR, whereas aspartic acid 96 plays a crucial role for the reprotonation of the Schiff base. Both residues are essential for an effective proton pump.

Key words: bacteriorhodopsin/proton pump/purple membrane

Introduction

The functional properties of bacteriorhodopsin (BR), the light-driven proton pump of *Halobacterium halobium* (Oesterhelt and Stoeckenius, 1973), have been studied in the past by a variety of biophysical methods. Optical spectroscopy demonstrated the photoisomerization of the retinylidene chromophore in the primary reaction and a sequence of dark reactions re-establishing the initial state. The intermediate products of this so-called photocycle have been designated J, K, L, M, N and O and have life-times in the femto- to millisecond range. The formation of the M-intermediate is accompanied by the deprotonation of the C=N double bond between the retinal moiety and the lysine residue 216 and release of the proton to the outer aqueous phase. Decay of the M-intermediate occurs under reprotonation of the chromophore and re-uptake of a proton from the aqueous phase. These are the key reactions by which vectorial proton transfer is linked to the photocycle of the molecule. Due to the electrogenic nature of proton translocation in BR the concomitant electrical phenomena were investigated in detail by electrical methods under both stationary and time-resolved conditions and could be correlated with the photocycle (Drachev *et al.*, 1974, 1976; Dancshazy and Karvaly, 1976; Herrmann and Rayfield, 1978; Keszthelyi and Ormos, 1980; Fahr *et al.*, 1981).

The proton transfer reactions in BR during the photocycle do not only involve changes of the retinal moiety but also protonation changes of amino acid side chains in the protein. Fourier transform infrared spectroscopy (FTIR) demonstrated that four aspartates undergo protonation changes, two of them being protonated in the initial state and two being deprotonated (Engelhard *et al.*, 1984; Eisenstein *et al.*, 1987). Site-specific mutagenesis of aspartic acids indeed indicated the importance of aspartic acid residues 85, 96, 115 and 212 for proper function (Mogi *et al.*, 1988). In an alternative approach to site-specific mutagenesis, mutation of halobacterial cells and selection of phototrophic-negative mutants allowed the isolation of bacterio-opsin point-mutations (Soppa and Oesterhelt, 1989). One advantage of this method is that BR becomes available in its natural lipid environment, the purple membrane. Two of these mutants are especially interesting: one carries an asparagine residue instead of aspartic acid at position 96 and in the other one aspartic acid 85 is replaced by a glutamic acid (Soppa *et al.*, 1989). The aspartic acid 96−asparagine exchange was investigated in detail by FTIR spectroscopy and allowed the identification of this residue as a protonated aspartic acid in the initial state that becomes deprotonated after formation of the M-state (Gerwert *et al.*, 1989). Here we show by detailed investigation of both mutated BRs that aspartic acid 96 is indeed involved in the reprotonation process of the Schiff base in BR and its lack causes retardation of the photocycle. Aspartic acid 85, on the other hand, seems to be involved in the deprotonation of the Schiff base and its exchange to glutamic acid accelerates the deprotonation reaction. A preliminary report on these results was presented at the XXI Yamada Conference on Retinal Proteins, June 1988, and has been published (Tittor *et al.*, 1989).

Results

Flash spectroscopy of mutated bacteriorhodopsins Asp^{85} → Glu and Asp^{96} → Asn

All experiments were carried out with BR isolated as purple membranes from *Halobacterium* sp. GRB (Ebert *et al.*, 1984). BR of this species has a primary structure identical to that of *H.halobium* (Soppa *et al.*, 1989) and purple membranes isolated from the cells show no difference to purple membranes from *H.halobium* on electron micrographs or in sucrose density gradients (data not shown). Initial control experiments with purple membranes from *H.halobium* and *Halobacterium* sp. GRB did not reveal any differences when the photocycle was measured with a time resolution of ~0.5 μs in gels or in aqueous solution.

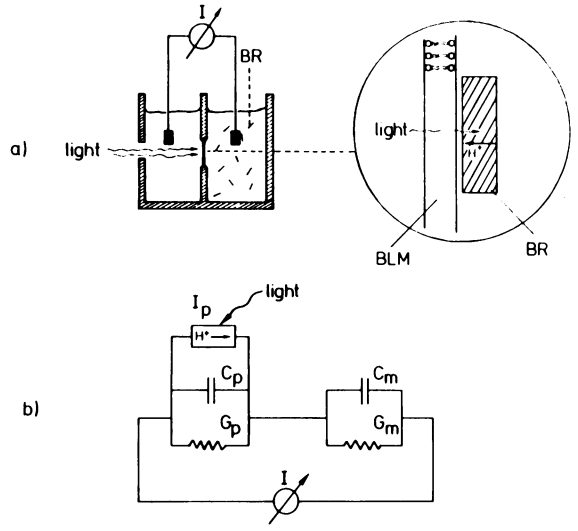

Fig. 1. (**a**) Experimental set-up for the measurement of light-induced photocurrents and schematic representation of the arrangement of purple membrane patches (BR) adsorbed to the black lipid membrane BLM. (**b**) Equivalent circuit scheme of the experimental system in (a). I_p is the pump current; C_p, C_m the capacity of the purple membrane and the underlying lipid film; G_p, G_m the conductance of the purple membrane and the underlying lipid film.

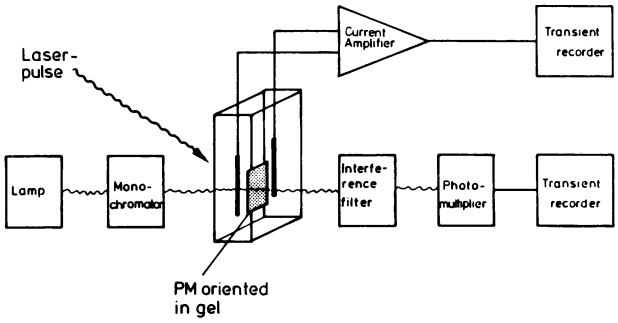

Fig. 2. Experimental set-up for electrical and absorption measurements on purple membranes oriented in a polyacrylamide gel.

Mutant strain 384 produces a BR that contains a glutamic acid in position 85 instead of the aspartic acid of the wild-type. The absorption spectrum of BR Asp^{85} → Glu at pH 6.5 is red-shifted, exhibiting a maximal absorbance at ~610 nm. Increasing the pH causes a decrease of absorption at 610 nm and an increasing dominance of shoulder at 550 nm (Figure 3). The results on the kinetics of the photocycle of BR Asp^{85} → Glu when excited by 540 nm laser pulses are shown in Table I. The M-formation is characterized by two relaxation times in wild-type BR of 50 and 150 μs, respectively. This has been interpreted as reflecting the formation of two different M-intermediates in a ratio of ~1:1 (F.Siebert, personal communication). In the case of BR Asp^{85} → Glu, two relaxation times of 5 and 150 μs at an amplitude ratio of ~9:1 were observed. The main component of M-formation in the mutant is accelerated by a factor of 10 compared to the corresponding time constant in the wild-type.

M-formation and decay of BR Asp^{85} → Glu show pH-independent kinetics between pH 5 and 9 within a factor of 2. It is especially important to note that the mutant protein behaves like the wild-type in the M decay.

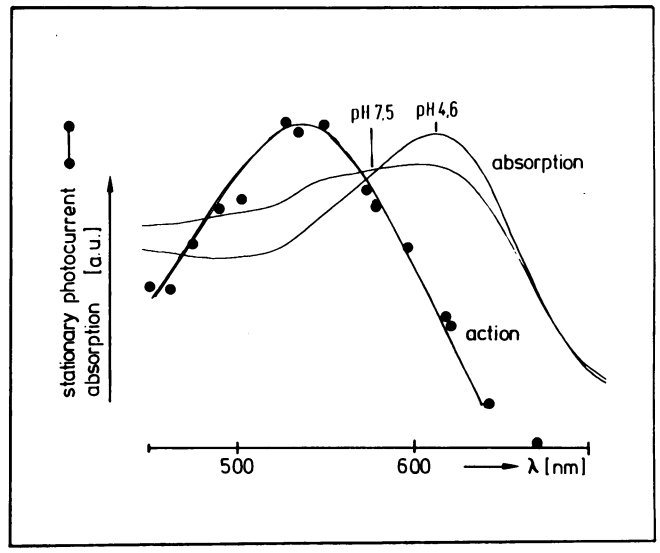

Fig. 3. Absorption spectra at pH 4.6 and 7.5 of BR Asp^{85} → Glu and action spectrum of the stationary photocurrents on planar lipid films in the mutant. Actinic light was varied by use of narrow band interference filters and measurements carried out in 100 mM NaCl at pH 6.5 (10 mM Mops buffer).

Mutant 326 contains a BR that carries an asparagine residue at position 96 of the amino acid sequence instead of an aspartic acid. The absorption spectrum of this mutated BR is identical to that of wild-type BR and M formation has the same kinetic characteristics within a factor of 2−3 as wild-type BR. The M decay, however, is drastically slowed down to 450 ms at pH 6.2 (Table I).

Photoelectrical experiments with the mutated bacteriorhodopsin Asp^{85} → Glu

The ion transport properties of BR and its mutated analogues were studied by measuring the displacement currents and the pump currents on purple membranes attached to planar lipid membranes and on purple membranes oriented and immobilized in polyacrylamide gel. In parallel, the above-mentioned spectroscopic experiments were repeated with samples immobilized in a gel matrix. The results showed identical spectroscopic behaviour of the BR molecules in both systems.

The absorption spectra of BR Asp^{85} → Glu at pH 4.6 and pH 7.5 are compared with its action spectrum in Figure 3. The action spectrum was determined by measuring the stationary photocurrents at different wavelengths using theT planar lipid bilayer system. Stationary currents were obtained after addition of the protonophore 1799 to permeabilize the planar lipid film underlying the purple membrane sheets, which causes a switch of the composed membrane system from an AC-coupled to a DC-coupled state. The action spectrum was also recorded by measuring stationary photocurrents from the gel-embedded oriented purple membrane sheets. Independently of pH the resulting photoresponse was maximal at 550 nm and thus confirmed the result shown in Figure 3 for the lipid membrane system. Obviously the shoulder at 550 nm in the absorption spectrum of BR Asp^{85} → Glu is due to a species that is active in proton pumping, but the main component absorbing maximally at ~610 nm is inactive in proton translocation.

The photocurrent kinetics of BR Asp^{85} → Glu were also

Table I. Spectroscopic and electric parameters of wild-type and mutated BR

Sample	Observables	Relaxation times (τ)/displaced charge (ΔQ)					
BR wild-type	absorption change at 419 nm (τ)			50 μs, increase	150 μs	3 ms, 8 ms decrease	
	photocurrent (τ)	< 150 ns	1.2 μs	50 μs	200 μs	4 ms	
	displaced charges (ΔQ) (nA × ms)	−1.8	−0.8	6	6	41	
						ms	pH
BR Asp^{96} → Asn	absorption change at 419 nm (τ)			100 μs	500 μs	450 ms	6.5
				increase		100 ms	4.3
						20 ms	2.7
						decrease	
	photocurrent (τ)	< 150 ns	1.1 μs	50 μs	300 μs	500 ms	
	displaced charges (ΔQ) (nA × ms)	−1.3	−0.6	6	15	~20[a]	
BR Asp^{85} → Glu	absorption change at 419 nm (τ)				150 μs	4 ms	15 ms
				increase		decrease	
	photocurrent (τ)	< 150 ns	400 ns	6 μs	150 μs	2 ms	16 ms
	displaced charges (ΔQ) (nA × ms)	−0.2	0.1	1.0	1.7	7	5

Measurements on wild-type BR and BR Asp^{96} → Asn were done in 10 mM Mops, pH 6.2, 20 mM KCl, those on BR Asp^{85} → Glu in 10 mM Tris, pH 8.4, 20 mM KCl unless otherwise specified. The time constant for the photocurrent in BR Asp^{96} → Asn was obtained from the plot of dark interval versus peak current, the displaced charges as described in the Discussion. For the mutant BR Asp^{85} → Glu, only time constants linked to the active species, i.e. in the millisecond range are given (see Discussion).
[a]For estimation of the displaced charges in this mutant see Discussion.

measured on the planar lipid film and in the gel-embedded preparation. The electrical events were initiated by a laser flash (540 nm, 10 ns pulse duration) and are shown in Figure 4. The overall shape of the time-resolved photocurrent signal is similar to that of the wild-type BR. A fast and negative current is followed by a positive charge displacement which has been assigned to the proton translocation during the steps L → M and M → BR ground-state of the catalytic cycle. The fast negative amplitude, on the other hand, was attributed to a conformational change within the protein, the *cis–trans* isomerization of retinal and some parasitaric charge movements within the protein during the initial photochemical steps and L-formation (Bamberg and Fahr, 1980; Keszthelyi and Ormos, 1980; Fahr *et al.*, 1981; Trissl and Gärtner, 1987). In BR Asp^{85} → Glu the positive signal of the photocurrent rises much faster than in the wild-type. As listed in Table I, the relaxation times of the mutant protein are decreased similarly as was measured for the photocycle kinetics. A time-constant of 50 μs for wild-type BR is found, but only of 6 μs for the mutant. A second kinetic component of 150 μs remains unaffected by the mutation. It is interesting that in addition to the relaxation time of 6 μs the mutant has another relaxation time of only 400 ns in its positive part replacing the negative 1.2 μs component of the wild-type. Control experiments showed that this relaxation times is indeed a property of the mutant protein and not due to an artefact of the measuring system. Further results of these experiments are summarized in Table I, which lists the relaxation times with their concomitant displaced charges. The main conclusion to be drawn is that replacement of Asp^{85} by Glu accelerates the electrogenic step linked to deprotonation of the Schiff base in BR.

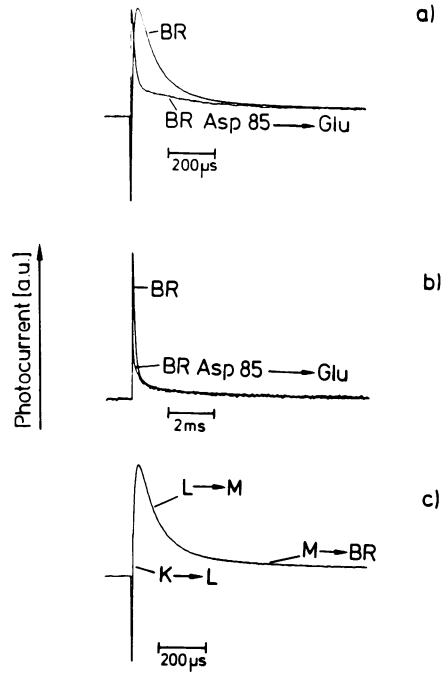

Fig. 4. Time-resolved current of BR Asp^{85} → Glu obtained with purple membranes oriented in a polyacrylamide gel in 20 mM KCl, 10 mM Tris, at pH 8.0. The wavelength of the laser light was adjusted to 540 nm for BR and to 570 nm for wild-type BR, respectively. The signals are averages of 15 measurements. (**a**) Photocurrent in the microsecond time range. (**b**) Photocurrent in the millisecond time range. (c) Indication of phases in the photocurrents of BR that could be correlated to spectroscopic transitions.

Photoelectrical experiments with the mutated bacteriorhodopsin Asp^{96} → Asn

In contrast to the mutation Asp^{85} → Glu, BR Asp^{96} → Asn has an action spectrum of ion translocation identical to its absorption maximum at 570 nm (not shown). Figure 5 compares the photocurrent under stationary illumination of purple membranes oriented in a polyacrylamide gel of the mutant Asp^{96} → Asn and the wild-type. Because the overall photocycle time of the mutant BR under the conditions applied is ~450 ms, a smaller photocurrent compared to the wild-type is expected. Therefore the concentration of

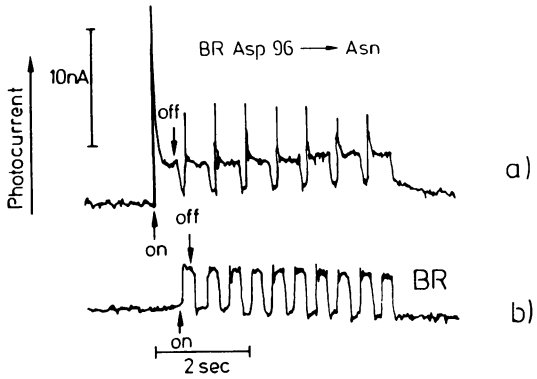

Fig. 5. Photocurrent of BR Asp^{96} → Asn (**a**), and wild-type BR (**b**) resulting from repetitive exposure to continuous light. Polyacrylamide gel, 10 mM Mes buffer, pH 6.0, and 20 mM KCl. Optical density for the mutant BR was 0.5 and for wild-type BR 0.02, respectively.

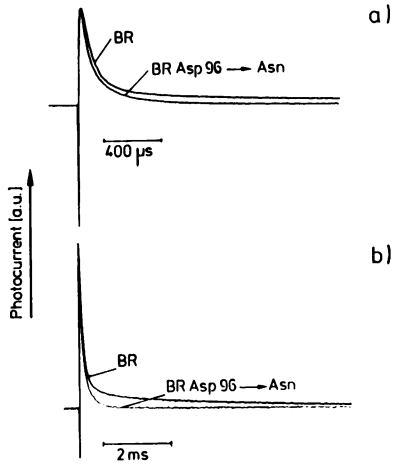

Fig. 6. Time-resolved current of BR Asp^{96} → Asn with purple membranes oriented in a polyacrylamide gel in 10 mM Mes buffer, pH 6.0, 20 mM KCl. Note the different time scales in (**a**) and (**b**).

wild-type BR in the gel was lowered to adjust its response to that of the mutant. Under these conditions the stationary currents of the two BRs are comparable, but a conspicuous difference exists in the shape of the electrical signals. After the onset of illumination, mutant Asp^{96} → Asn shows a peak current which decays to a stationary value (Figure 5a) while the signal from the wild-type sample lacks this peak current (Figure 5b). The height of the peak in the mutant preparation is dependent on the length of the dark period between two subsequent illuminations. In Figure 5 two different peak values, after a very long dark interval (first illumination) and after intermediate dark times (all other illuminations) are shown. Shortening or extending the dark interval modulates the peak current from an almost negligible amplitude at very short intervals to a maximum peak height at very long dark intervals. By plotting the interval time against the corresponding peak height the exact value of its recovery time could be obtained. A value of 500 ms was determined, reflecting the cycle time of the mutant (see Discussion).

The kinetics of the photocurrent of mutant BR Asp^{96} → Asn were also measured after a laser flash and correlated with its photocycle kinetics. Comparing the mutant with the wild-type, the fast part of the kinetics up to M-formation is changed by at most a factor of 3. A drastic effect, however, is that no current relaxation occurs in the millisecond range (Figure 6b). Since the decay of M in the mutant increases to 450 ms, no direct observation of electric relaxation is possible because the amplitude of the current during this process is too small. This does not mean, however, that no proton transport occurs in the mutated BR. As already demonstrated by the stationary current, such transport must occur and an evaluation of displaced charge upon M decay is presented in the Discussion.

Further evidence for the long cycling time is obtained by the following experiments. Spectroscopic and electric transport measurements showed that the catalytic cycle time of BR Asp^{96} → Asn was increased by a factor of 50 at pH 6 compared to BR. This implies that a different saturation behaviour with respect to the intensity of the incident light as energy-providing substrate should be observed. Indeed, already at an irradiance of 50 mW/cm^2 the stationary pump current of the mutant saturates, whereas wild-type BR has still not reached its saturation at 2 W/cm^2 (Figure 7). The interpretation of both the low stationary current and the ease of light saturation of the photocurrent has to be due to the only apparently changed rate constant in the catalytic cycle: the M decay.

The blue light effect of the M-intermediate in bacteriorhodopsin Asp^{96} → Asn

Another type of experiment also justifies this conclusion. It is known that the M-intermediate of BR can be photochemically reconverted to the initial state by illumination with blue light. Spectroscopic data showed that in a photostationary state mixture of M and BR ground-state additional blue light illumination diminishes the concentration of M (Oesterhelt and Hess, 1973). In experiments with BR-doped planar lipid membranes the development of a photopotential by illumination with green light was quenched by additional illumination with blue light (Karvaly and Dancshazy, 1977; Ormos *et al.*, 1978). The results of these experiments clearly indicated that photochemical conversion of M to the BR ground-state is not an acceleration of the pump cycle of BR, but in contrast a short circuit of this cycle preventing proton pumping. The observed electrical signal after excitation of the M-intermediate by short blue light laser pulses was interpreted as due to retinal re-isomerization, a resulting conformational change of the protein and proton re-uptake from the side of the molecule to which the proton was released during M formation (Ormos *et al.*, 1978).

Thus, the blue light effect of BR was used to prove that the extreme saturation behaviour of the stationary photocurrent in the mutant Asp^{96} → Asn is due to accumulation of the M-intermediate. In Figure 8 the behaviour of the mutant and the wild-type protein is shown. In Figure 8a the identical behaviour of mutant and wild-type protein upon flash photolysis in the time domain before the formation of the M-intermediate is evident. Correspondingly, as shown in Figure 8b, a blue laser flash (408 nm) does not produce an electric signal in either of the samples except as a small artefact. If the same experiment is repeated in the presence of green background light a drastic difference occurs between the wild-type and the mutant protein (Figure 8c). A much larger negative signal is observed for the mutant protein, indicating a higher steady-state concentration of M compared

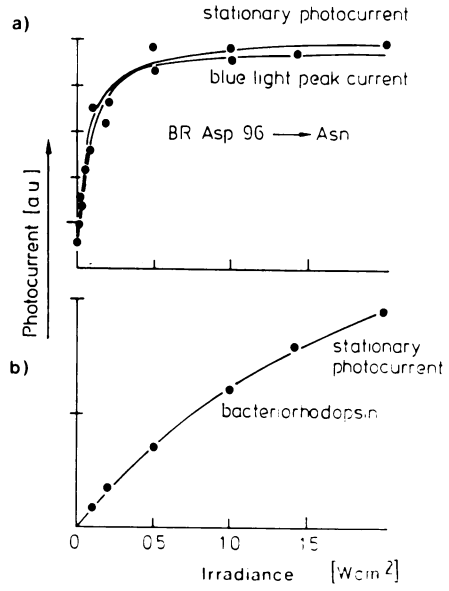

Fig. 7. Dependency of the stationary photocurrent on light intensity for mutant Asp^{96} → Asn (**a**) and wild-type BR (**b**) in 20 mM KCl, 10 mM Mes at pH 6.0. The photocurrents determined on planar lipid membranes and on the gel preparation were the same, but only the experiments obtained with the gel preparation are shown. For description of the blue-light-induced peak current see text and Figure 8.

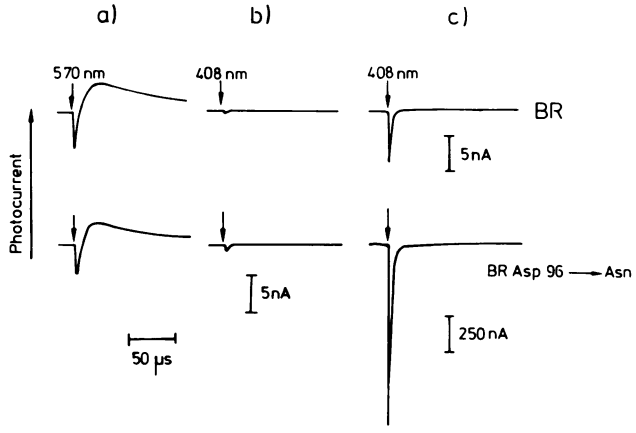

Fig. 8. Blue light effect on BR Asp^{96} → Asn and on wild-type BR with and without background light $\lambda \geq 496$ nm in a gel preparation at pH 6.0 (20 mM KCl, 10 mM Mes). (**a**) Photocurrent of BR Asp^{96} → Asn and wild-type BR after a 570-nm laser pulse. (**b**) Photocurrent after a 408-nm laser pulse. (**c**) Photocurrent of wild-type BR in the presence of 500 mW/cm² background light ($\lambda \geq 490$ nm) after a 408-nm laser pulse and BR Asp^{96} → Asn, but with 50 mW/cm² background light. Note the different scales for the photocurrent.

to the wild-type at the same background intensity of green light. A quantitation of this effect is obtained by plotting the height of this negative blue-light-induced signal versus the background light intensity (Figure 7). The saturation of the blue-light-induced electric signal is in good agreement with the saturation behaviour of the stationary pump current. This demonstrates that the exchange of aspartic acid 96 for an asparagine limits the photocycle by slowing down the electrogenic proton transfer from the external aqueous phase to the unprotonated Schiff base in the M-state.

Discussion

The two point-mutated BRs described in this paper were originally isolated from two mutant halobacterial strains unable to grow phototrophically (Soppa *et al.*, 1989). Both affect the de- and reprotonation of the Schiff base during the catalytic cycle of the proton pump mutations. The same conclusion was reached by systematic variation of aspartates in BR through site-specific mutagenesis (Mogi *et al.*, 1988). In this approach BR is expressed in *E.coli*, after genetic manipulation isolated in denatured form and reconstituted *in vitro* with retinal. In the approach described here, BR is expressed in halobacteria and isolated in its natural lipid environment, i.e. the purple membrane. As expected, some differences were observed. BR Asp^{85} → Glu isolated from halobacteria shows a pH-dependent spectrum created by two species: one inactive and absorbing at 610 nm and the other absorbing at ~550 nm and showing photoelectrical activity. Isolated from *E.coli*, the same mutant had an absorption maximum in CHAPS−SDS of 560 nm and in dodecylmaltoside of 610 nm (Mogi *et al.*, 1988). The 560 nm species showed proton pumping activity and might be similar to the 550 nm component that we observe in purple membranes. Indeed, detergent treatment removes the 610 nm species in our samples and shifts the absorption maximum to 560 nm. A more detailed spectroscopic analysis will be necessary to understand the influence of the mutation Asp^{85} → Glu with respect to structural effects that secondarily influence function.

The mutation BR Asp^{96} → Asn affects the reprotonation of the C=N double bond, thus decreasing the decay rate of M, which also becomes pH dependent (see Table I). The electric component corresponding to M decay is no longer seen in the laser-flash-induced photocurrents and we used different approaches to demonstrate the electrogenic nature of the reprotonation reaction of the Schiff base in the mutant. Stationary photocurrents of the mutant demonstrated a small but measurable pump activity. The initial peak current preceding the stationary photocurrent (Figure 5) also shows a slow cycling BR. This is based on the following consideration. Relaxations of wild-type and mutant BR to a steady-state current are described by the equation

$$\frac{1}{T} = \frac{1}{T_M} + \frac{1}{T_L}$$

with T being the relaxation time to reach the stationary current level, T_M the decay time of the intermediate M and T_L the time constant for M-formation. $1/T_L$ is the product of the light intensity, the quantum yield and the extinction coefficient (Fendler *et al.*, 1987). Under the experimental conditions of Figure 5 and the given time resolution in the experiment (3 ms), the relaxation of wild-type BR to the photo steady state is dominated by $1/T_M$, which is faster than the experimental time resolution. In the mutant, on the other hand, $1/T_M$ becomes so small due to the 500 ms time constant of M decay that $1/T_L$ dominates and the relaxation to the photostationary current is correspondingly slow enough to allow observation of the peak current before the stationary state level is reached. If light is turned off, the stationary state concentration of M decreases and, depending on the dark period between two subsequent illuminations, different concentrations of M remain. This allows the determination of the M decay time constant, which

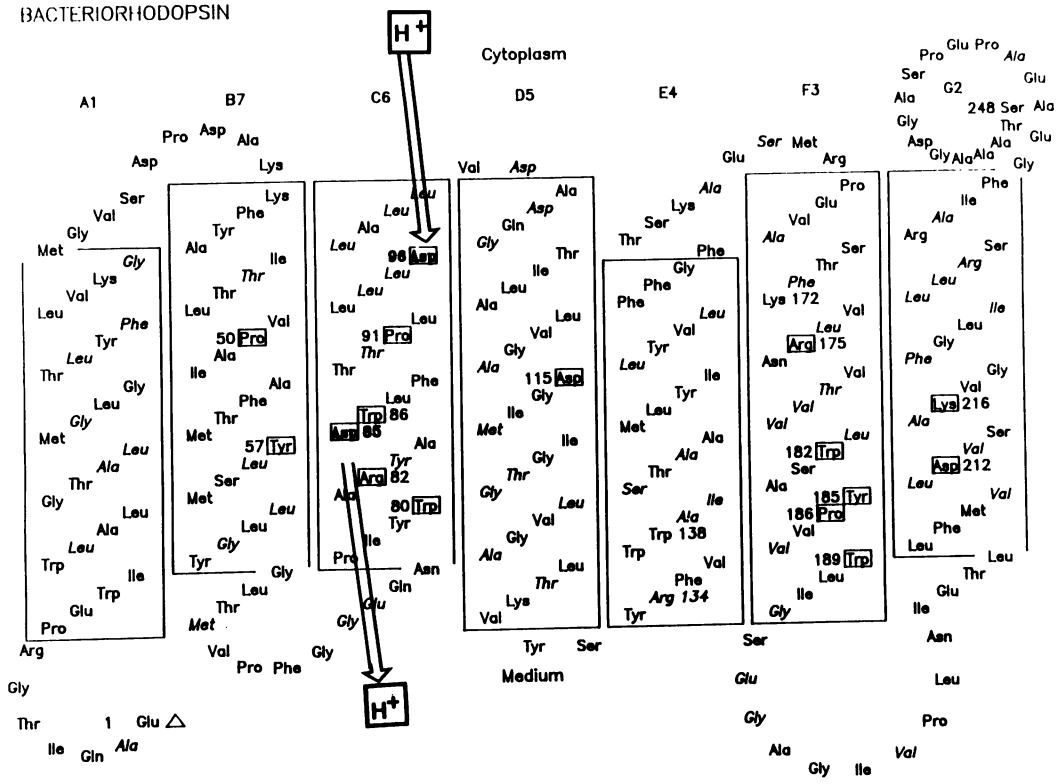

Fig. 9. Possible secondary structure of BR with transmembrane sections shown as seven blocks. The substituted aspartates 96 and 85 are shown in bold print. Other residues of possible functional importance and conserved between BR and halorhodopsin are also boxed.

was found to be 500 ms and corresponds well with direct spectroscopic measurements. To demonstrate the electrogenic nature of the M decay in the mutant the displaced charges ΔQ (Table I) during this transition were estimated even though they were not directly observable in the experiment of Figure 6. The results of the experiments in the presence of green background light (Figures 7 and 8) allow a calibration of the displaced charges during the formation of M in the mutant compared to the wild-type. Together with the known concentration of M, the charge displacement during M decay in the mutant can be evaluated. About the same charge displacement for M formation and M decay is found, a ratio that is similar to that in the wild-type.

Secondary structural predictions locate aspartic acid residues 85 and 96 in the transmembrane helix C of BR wild-type (Figure 9) in such a way that aspartate 85 is closer to the outer surface and 96 closer to the inner surface of the molecule. This would fit their postulated role in mediating proton transfer from the Schiff base to the medium and from the cytoplasm to the Schiff base. Two more intramembrane aspartates in positions 115 and 212 occur and are conserved in halobacterial proteins (Blanck and Oesterhelt, 1987). It is a matter of conjecture whether these two residues also participate in the proton transfer pathway from and to the Schiff base or only mediate configurational and conformational changes of the retinal or the protein moiety. By FTIR experiments it was shown that Asp^{212} and Asp^{85} are in a deprotonated form in BR wild-type while Asp^{115} and Asp^{96} are protonated (Braiman *et al.*, 1988; Gerwert *et al.*, 1989). This leaves the interesting possibility that BR $Asp^{85} \rightarrow$ Glu consists of a mixture of a protonated residue 85, which absorbs at 610 nm, and a deprotonated residue, as in the wild-type, absorbing at ~550 nm and being active. Acidification of BR wild-type, as has been known for a long time, also leads to a blue inactive form of BR and might also involve Asp^{85}.

Aspartate 96 is a suitable candidate for reprotonation of the Schiff base, either directly or via participation of other residues. The protonated Asp^{96} residue in the interior of the membrane explains the essential pH independence of the M decay in wild-type BR and the dramatic increase in rate at low pH upon removal of the internal proton donor. Certainly it will be interesting to investigate in detail isotope effects and spectroscopic properties of intermediates following M in these mutants. Taken together, the study of point-mutated BRs that are defective in the proton pump seem to be extremely useful in studying its function.

Materials and methods

Halobacterium sp.

GRB mutant strains 326 and 384 (Soppa and Oesterhelt, 1989) were used for isolation of purple membranes (Oesterhelt and Stoeckenius, 1974). Purple membranes isolated from mutant strain 326 contain bacteriorhodopsin in which aspartate 96 is exchanged for an asparagine. Mutant strain 384 carries a glutamic acid residue instead of aspartic acid at position 85. The primary structure of bacteriorhodopsin from the wild-type *Halobacterium* sp. GRB is identical to that from *Halobacterium halobium* (Soppa *et al.*, 1989). The reason for using *Halobacterium* sp. GRB for mutagenesis and selection of mutants was its higher genetic stability compared to *H.halobium*.

Electrical transport studies were performed with purple membranes adsorbed on one side of the planar lipid membrane and with purple membranes oriented in an electrical field and subsequently immobilized in a polyacrylamide gel (Eisenbach *et al.*, 1977; Dér *et al.*, 1985).

Preparation of the planar lipid bilayers

Optically black lipid membranes with an area of $\sim 10^{-2}$ cm^2 were formed in a Teflon cell filled with an appropriate electrolyte solution (1.5 ml in each compartment). The membrane-forming solution contained 1.5% (w/v) diphytanoyl lecithin (Avanti Biochemicals, Birmingham, AL) and 0.025% (w/v) octadecylamine (Riedel-de-Haen, Hannover, FRG) in *n*-decane to obtain a positively charged surface (Dancshazy and Karvaly, 1976). Purple

membrane suspensions (OD_{568} = 10) were sonicated for 1 min in a sonication bath and aliquots of 20 μl added under stirring to the rear compartment (Figure 1a). Photosensitivity of the samples developed in time and reached their maximal value after ~40 min. The membrane was illuminated either with a 250 W tungsten light source and filtered through appropriate filters or by light from a dye laser (Lambda Physics, 10 ns pulse length tuned to the appropriate wavelength). Light reached the sample after passing through the front compartment. The irradiance of the continuous light source was up to 2 W/cm^2 when filtered through heat-protecting glass and a 495 nm cut-off glass filter. The laser light pulse energy was varied between 0 and 500 μJ. For recording action spectra light was filtered through narrow-band interference filters (band width 10 nm, Schott, Mainz, FRG).

The membrane cell was 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) in stationary experiments, whereas for the kinetic measurements a home-made current–voltage converter was used. The time resolution in the kinetic experiments was ~1–2 μs. The purple membrane and the planar film are capacitively coupled so that

$$I_0 = I_{p0} \frac{c_m}{c_m + c_p}$$

where I_0 is the initial current at time 0 and I_{p0} the pump current in the stationary state at zero potential (Figure 1b). Further details of this system are described in Fahr *et al.* (1981).

Acrylamide gels containing oriented purple membranes

The purple membranes from wild-type and mutant cells were oriented in an electric field and immobilized by polymerization of acrylamide contained in the sample according to Eisenbach *et al.* (1977) and Dér *et al.* (1985). Control experiments were carried out with the same purple membrane suspension immobilized in agar gel and the photoresponse was found to be the same as in the polyacrylamide gel. Gel pieces of 1.3 × 8 × 7 mm were cut and bathed for 3 days in electrolyte solution. Platinized platinum electrodes protected from incident light were immersed on both sides of the gel in the bathing solution. The cuvette was shielded in a thermostatted Faraday cage with a 7 × 7 mm window for the incident light. In kinetic experiments the sample was excited with the appropriate wavelength by a dye laser pulse of 10 ns duration and an energy of up to 500 μJ. The light pulse reached the sample through an optic fibre at an angle of 45° from above. The experimental set-up is shown in Figure 2. Current amplifiers of different time resolutions (0.15, 1 and 100 μs) were connected successively to the electrodes in order to obtain a high signal-to-noise ratio for each time range. Absorption changes in the samples were monitored with monochromatized light from a 250 W halogen tungsten lamp, which then passed an interference filter before its intensity was measured by a photomultiplier. The interference filter behind the sample protects the photomultiplier from the laser flash. The kinetics of the photocycle and of the photocurrent were recorded simultaneously in two channels of a transient recorder. After each flash 128 000 data points were collected in each channel. The data were sampled at a constant rate of 0.2 μs/point and then compressed by averaging over blocks of sampled data points. The block length increased from the beginning to the end of the recording period so that two data sets of 684 points in a quasi-logarithmic time-scale were obtained, which had intervals of, for example, 0.2 μs at the beginning and 410 μs at the end of each data set. All signals obtained were fitted with a sum of exponentials.

Chemicals

1799, a UV and blue-light-insensitive protonophore (2,6-dihydroxy)-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptane-4-one and FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone) were kindly provided by Dr P.Heytler (Du Pont de Nemours and Co.).

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