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Decoupling of photo- and proton cycle in the Asp85 → Glu mutant of bacteriorhodopsin

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Surface bound pH indicators were applied to study the proton transfer reactions in the mutant Asp85 → Glu of bacteriorhodopsin in the native membrane. The amino acid replacement induces a drastic acceleration of the overall rise of the M intermediate. Instead of following this acceleration, proton ejection to the extracellular membrane surface is not only two orders of magnitude slower than M formation, it is also delayed as compared with the wild-type. This demonstrates that Asp85 not only accepts the proton released by the Schiff's base but also regulates very efficiently proton transfer within the proton release chain. Furthermore, Asp85 might be the primary but is not the only proton acceptor/donor group in the release pathway. The Asp85 → Glu substitution also affects the proton reuptake reaction at the cytoplasmic side, although Asp85 is located in the proton release pathway. Proton uptake is slower in the mutant than in the wild-type and occurs during the lifetime of the O intermediate. This demonstrates a feed-back mechanism between Asp85 and the proton uptake pathway in bacteriorhodopsin.

Key words: bacteriorhodopsin/pH indicator/proton transfer/purple membrane/site-specific mutagenesis

Introduction

Protons play a central role in bioenergetics. Their importance is expressed by Mitchell's chemiosmotic hypothesis. With bacteriorhodopsin (BR), the proton translocating protein of halobacteria (*Halobacterium salinarium*), proton transfer steps can be investigated at almost atomic level although the three-dimensional structure of BR has not yet been determined at this resolution. BR is composed of 248 amino acids and a retinal molecule which is linked to Lys216 via a protonated Schiff's base. The polypeptide traverses the membrane seven times as α-helices. Light absorption by BR induces a photocycle with various intermediates (J, K, L, M, N and O) having characteristic absorption maxima and rise times (for a recent review see Mathies *et al.*, 1991). The M intermediate is crucial for proton translocation through BR because it is the only intermediate with a deprotonated Schiff's base. It has been shown by application of covalently bound pH indicators that the translocated proton appears at the extracellular surface concurrent with the rise of the M intermediate (Heberle and Dencher, 1990, 1992a,b; Dencher *et al.*, 1991).

The introduction of point mutated BR is a very promising approach towards unravelling the role of specific amino acids in proton transfer within the protein. With this tool it was demonstrated that several amino acid residues are necessary for proper function of the proton pump. Namely, the aspartic acids 85, 96, 115 and 212 as well as Arg82 seem to be involved in intraproteinous proton transfer (Butt *et al.*, 1989; Otto *et al.*, 1990; Thorgeirsson *et al.*, 1991).

In order to gain further insight into the primary steps of proton translocation, we have studied the effect of replacing Asp85, which is the primary acceptor of the Schiff's base proton (Fahmy *et al.*, 1992; Metz *et al.*, 1992) by Glu, i.e. of an increase in length of the side-chain by one methylenic group. The *in vivo* mutated Asp85 → Glu BR in *Halobacterium* sp. GRB (Soppa and Oesterhelt, 1989) exists as a pH-dependent mixture of two blue (absorption maxima at ~615 nm) and two red (532 nm) chromophoric states (in separate equilibria with pK_a of 4.6 and 9.5, respectively; Lanyi *et al.*, 1992). Glu85 is protonated in the blue states, and deprotonated in the red states (Fahmy *et al.*, 1992). The mutated Asp85 → Glu BR is arranged in the characteristic two-dimensional hexagonal lattice of the purple membrane (PM). There are only minor differences in the X-ray diffraction pattern between the red forms of the mutated BR and the wild-type (Lanyi *et al.*, 1992; G.Büldt, N.A.Dencher and M.H.J.Koch, unpublished results). Only the red chromophoric state translocates protons (Butt *et al.*, 1989; Subramaniam *et al.*, 1990) and was therefore investigated in detail by time-resolved flash spectroscopy and pH indicators selectively bound to Lys129 at the extracellular surface of BR. Our results demonstrate that Asp85 plays an essential role in the proton release reaction from the Schiff's base to the extracellular membrane surface and that at least one additional proton donor/acceptor group is present in the release pathway. For the first time a decoupling of the ion pumping cycle from the photocycle could be observed. Upon replacement of Asp85 by Glu, the simultaneousness of M intermediate formation and proton ejection to the extracellular membrane surface (Heberle and Dencher, 1990, 1992a,b) is no longer sustained, but the latter process is delayed by two orders of magnitude. Also the proton reuptake pathway is affected and proton uptake occurs during the O state.

Results

Fluorescein bound to Lys129 at the extracellular surface of BR and pyranine residing in the aqueous bulk phase monitor proton ejection to the membrane surface and subsequent proton transfer into the aqueous environment, respectively (Heberle and Dencher, 1990, 1992a,b; Grzesiek and Dencher, 1986). Due to the pK value of these pH indicators, measurements are best performed in the pH range between

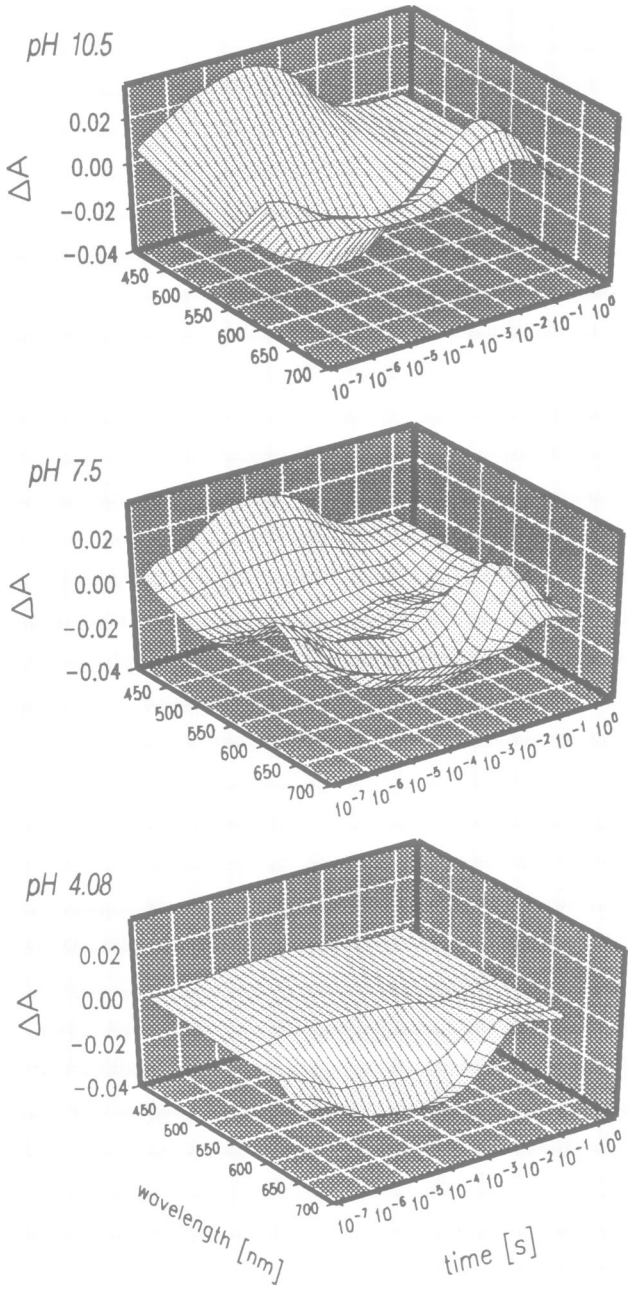

Fig. 1. Three-dimensional plot of the photocycle of the Asp85 → Glu mutant at pH 10.5, 7.5 and 4.08 (from top to bottom). The transient changes in absorbance after a nanosecond laser pulse were recorded at different wavelengths. Traces at constant wavelength represent multiexponential fits to the data. Data points at equal times are connected by straight lines. The middle panel (pH 7.5) depicts data traces measured at 410, 430, 450, 470, 490, 511, 520, 550, 570, 590, 610, 630, 650, 670 and 690 nm. The upper and lower panels represent data obtained at 412, 550, 610, 630 and 650 nm (T = 20°C, 150 mM KCl).

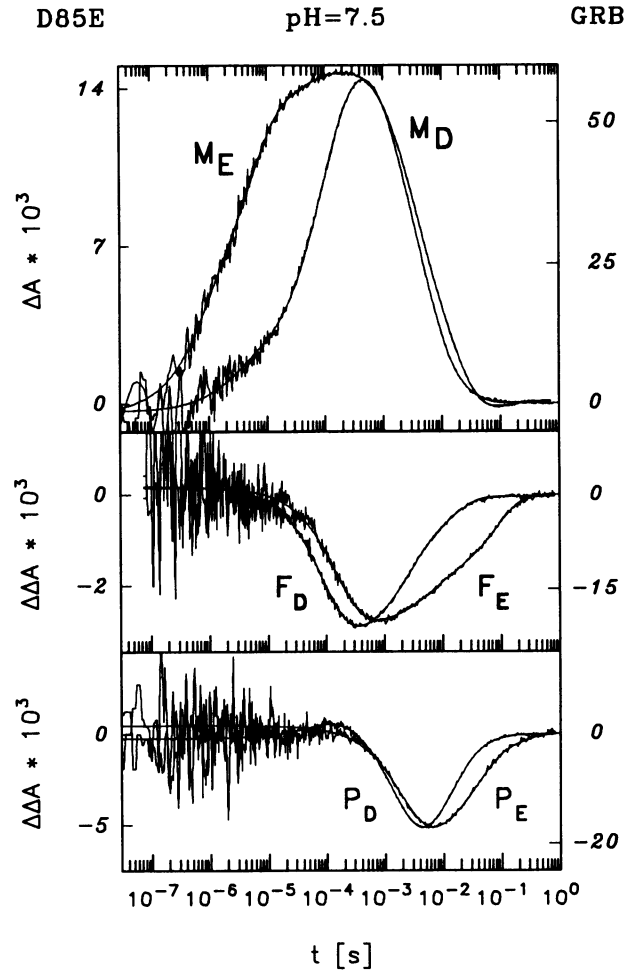

Fig. 2. Transient absorbance changes of the M intermediate in the Asp85 → Glu mutant (M_E) and in the wild-type BR (M_D) at pH 7.5, and the corresponding proton transfer reactions detected by surface bound fluorescein (F_E and F_D, respectively), and pyranine (P_E and P_D) dissolved in the bulk water phase. (T = 20°C, [GRB] = [D85E] = 11 μM; [F_D] = [F_E] = 6 μM; [P_D] = [P_E] = 40 μM; [KCl] = 150 mM; λ_M = 412 nm; λ_F = 489 nm; λ_P = 457 nm.) The left ordinate represents absorbance changes obtained with the mutant (D85E). The right ordinate corresponds to the wild-type (GRB). Smooth lines represent multiexponential fits to the data traces ($\Delta A = \Sigma A_i * e^{-t/\tau_i}$, where ΔA is the change in absorbance, and A_i and τ_i are the amplitude and time constant of the exponential function). Fit results are depicted in Table I.

6.8 and 7.8. Although at pH 7.5, where we have performed most of the measurements and where the Asp85 → Glu mutant BR exists as a mixture of blue (absorption maximum at ~615 nm) and red (532 nm) chromophoric states (Butt *et al.*, 1989; Lanyi *et al.*, 1992), the detected absorbance changes are to a large extent caused by the red state active in proton pumping (Figure 1, middle contour plot). This is due to the laser excitation wavelength of 532 nm, selecting predominantly the red chromophoric state. Supporting this statement, similar spectroscopic features occur at pH 10.5, where almost exclusively the red state is present (Figure 1, upper contour plot), and at pH 7.5 (Figure 1, middle contour plot). In agreement with previous investigations (Butt *et al.*, 1989; Lanyi *et al.*, 1992), a wild-type-like photocycle is observed for the red chromophoric state. At variance to the wild-type, the formation rate of the M intermediate is strongly increased in the mutant (Figure 2, upper panel). Formation and decay of the O intermediate (Figure 1) are strongly retarded (τ_f = 22 ms, τ_d = 170 ms in the mutant as compared with τ_f = 1.9 ms, τ_d = 13 ms in the wild-type). We assign the pronounced positive peak around 620 nm in the millisecond time domain (Figure 1, upper and middle panel) to the O intermediate (in accordance with Fahmy *et al.*, 1992) and not to a red-shifted N-like intermediate (Lanyi *et al.*, 1992). The N intermediate can be detected at pH 10.5 as a slow component in the ground state recovery kinetics (hidden in upper panel of Figure 1),

Table I. Kinetic parameters of wild-type and Asp85 → Glu bacteriorhodopsin

		Wild-type						D85E mutant						
M	τ	2.7 μs	73 μs	231 μs	1.4 ms	5.4 ms	22 ms	735 ns	5.4 μs	32 μs	283 μs	2.7 ms	15 ms	159 ms
	A	−11%	−47%	−42%	28%	59%	13%	−29%	−49%	−11%	−11%	48%	54%	−2%
F	τ		92 μs prot.		2.3 ms deprot.		15 ms deprot.			157 μs prot.		5.3 ms deprot.	83 ms deprot.	
P	τ		−		1.6 ms prot.		13 ms deprot.			−		1.9 ms prot.	51 ms deprot.	

Comparison of the kinetics of the M intermediate (M) and the pH indicators fluorescein (bound to the surface of BR) (F) and pyranine (dissolved in the bulk water phase) (P), respectively (for experimental data and conditions see Figure 2). τ and A are the time constants and amplitudes, respectively, of the fitted exponentials; prot. and deprot. are the protonation and deprotonation reaction of the respective pH indicator.

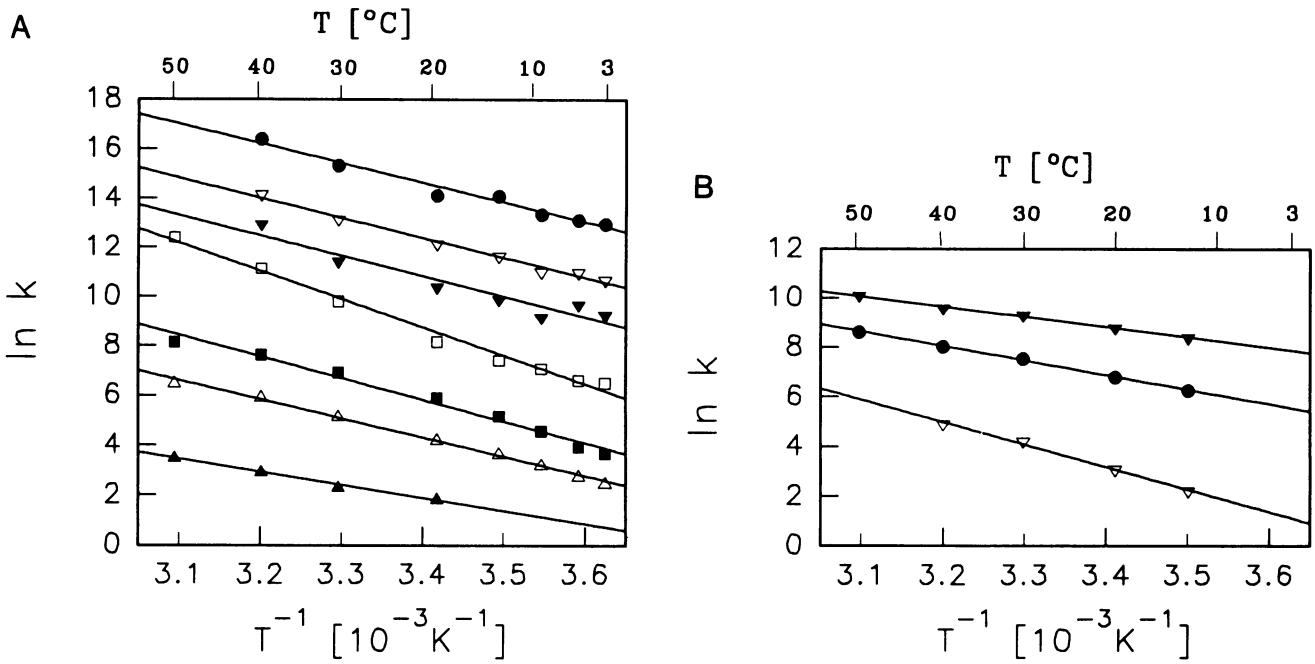

Fig. 3. Semilogarithmic plot of the rate-constants, k_i, versus the reciprocal temperature (Arrhenius diagram) for the kinetics of (**A**) the M intermediate in the Asp85 → Glu mutant [●, k_1 (E_a = 65.7 kJ/mol); ▽, k_2 (E_a = 67.1 kJ/mol); ▼, k_3 (E_a = 69.1 kJ/mol); □, k_4 (E_a = 95.6 kJ/mol) for the M rise and ■, k_5 (E_a = 72.2 kJ/mol); △, k_6 (E_a = 63.7 kJ/mol) for the decay kinetics. ▲, k_7 (E_a = 82.7 kJ/mol), is the rate constant of an additional exponential that has a small positive amplitude] and (**B**) proton transfer reactions: ▼, intraproteinous proton translocation to the membrane surface (E_a = 35.0 kJ/mol) detected by surface bound fluorescein; ●, surface/bulk transfer (E_a = 48.5 kJ/mol) and ▽, subsequent proton uptake (E_a = 75.1 kJ/mol) detected by pyranine in the bulk water phase.

and even more pronounced when the mutant is solubilized in Triton X-100 (data not shown). In the mutant the O intermediate is transiently accumulated to a larger extent than in the wild-type. The photocycle of the blue chromophoric state can be clearly distinguished from that of the red state by the absence of the M and O intermediates (Figure 1, lower contour plot).

The upper traces of Figure 2 represent rise and decay of the photocycle intermediate M in the Asp85 → Glu mutant (M_E) and in wild-type BR (M_D). The yield of M in the Asp85 → Glu mutant is only 25% of the yield in the wild-type at this pH. It is quite obvious from Figure 2 that the rise of M, reflecting deprotonation of the Schiff's base, is drastically accelerated in the mutant as compared with the wild-type, i.e. half of the signal amplitude is reached after about 2 μs as compared with 60 μs. Four exponentials are required to fit the M rise satisfactorily (Table I, Figure 3A). The decay of the M intermediate is slightly slower in the mutant. The decay is described by two exponentials. However, after M has completely disappeared a small negative component is observable which increases in amplitude at higher temperatures. According to the time constant, it can be assigned to the decay of the O intermediate. It should be noted that using a rhodamine 6G dye laser (λ = 580 nm) as excitation source instead of the Nd:YAG laser, does not significantly alter the time-course of the M intermediate.

The temperature dependence of the M intermediate kinetics was studied between 3 and 50°C (Figure 3A). The calculated activation energies for the mutant are comparable to those determined for the wild-type (Heberle and Dencher, 1992b). This indicates that the general mechanism of the photocycle is not altered for the red chromophoric state of the mutant.

As demonstrated in a previous study on wild-type BR, proton release kinetics can only be determined with a pH

indicator covalently bound to the extracellular side of BR (Heberle and Dencher, 1990, 1992a,b). A derivative of fluorescein (FITC) that is selective for free amino groups, was covalently linked to Lys129 at the extracellular surface of BR yielding a stable thiourea. The middle panel in Figure 2 depicts the absorbance changes of the surface bound fluorescein in wild-type (F_D) and in the Asp85 → Glu mutant (F_E). At the absorbance maximum of fluorescein (λ = 489 nm), a decrease in absorbance corresponds to the protonation of the pH indicator, i.e. the pH in the vicinity of the indicator is lowered. From the time-course of the response of fluorescein bound to wild-type BR (F_D in Figure 2), it is obvious that the proton is pumped from the protein interior to the surface during the rise of the M intermediate. In the Asp85 → Glu mutant the rise of the M intermediate is strongly accelerated (Table I). Hence, the question arises if the proton transfer within the protein follows this acceleration. As monitored by surface bound fluorescein, appearance of the translocated proton at the extracellular side of the mutated BR (F_E in Figure 2) is not only two orders of magnitude later than the overall rise of the M intermediate (M_E), but even slower in the mutant than in the wild-type. The proton is released to the mutant's membrane surface with a time constant of τ = 157 μs at 20°C. Therefore, the exchange of Asp85 with Glu leads to a retardation of the proton inside the protein by a factor of 1.7 (wild-type: τ = 92 μs, see Table I). The proton reuptake reaction monitored by both the slower component of the deprotonation reaction of fluorescein and by the deprotonation of pyranine, proceeds 5.5 and 3.9 times, respectively, slower in the mutant than in the wild-type (Figure 2, Table I). Furthermore, the temporal correlation of proton uptake with the slowest component of the M decay in the wild-type is not present in the mutant.

The activation energies of the protonation reaction of surface-bound fluorescein (E = 35.0 kJ/mol, reflecting proton ejection to the membrane surface) and of pyranine [E = 48.5 kJ/mol, reflecting proton transfer from the membrane surface into the aqueous bulk phase (Heberle and Dencher, 1990, 1992a,b)] are the same within experimental error for the mutant and the wild-type. In contrast, the activation energy of proton uptake (deprotonation of pyranine) is elevated in the mutant, i.e. E_a = 75.1 kJ/mol (Figure 3B) as compared with 58.8 kJ/mol in the wild-type (Heberle and Dencher, 1992).

As mentioned before, the red species of the Asp85 → Glu mutant has a low concentration at pH 7.5. Increasing the pH leads to higher amounts of this ion-pumping active species. pH indicators have only a limited detection range, usually pH = pK ± 1. To measure the proton transfer reactions in the mutant at elevated pH values, we used instead of fluorescein a derivative of coumarin [3-(2-benzothiazolyl)-7-hydroxycoumarin-4-carboxylic acid succinimidyl-ester, BHCCSE]. Instead of pyranine, the derivative 1,3-dihydroxy-6,8-pyrenedisulfonate was employed. The time-resolved absorbance changes of the M intermediate and the corresponding proton transfer kinetics monitored by surface bound BHCCSE of the Asp85 → Glu mutant at pH 8.6 are depicted in Figure 4 and compared with the wild-type. For wild-type BR the M rise is faster at alkaline pH than at pH 7.5. The mutant does not exhibit such pH-dependence of the M rise (see also Lanyi *et al.*, 1992). The appearance of the pumped protons at the membrane surface

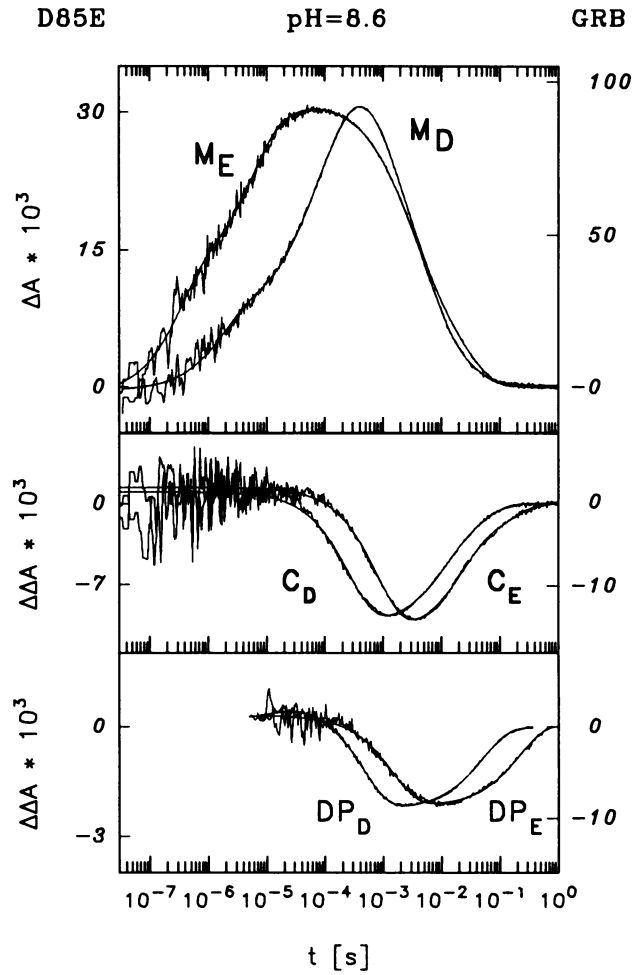

Fig. 4. Transient absorbance changes of the M intermediate in the Asp85 → Glu mutant (M_E) and in the wild-type BR (M_D) at pH 8.6, and the corresponding proton transfer reactions detected by surface bound 3-(2-benzothiazolyl)-7-hydroxycoumarin (C_E and C_D, respectively) and 1,3-dihydroxypyrene-6,8-disulfonate (DP_E and DP_D) dissolved in the aqueous bulk. (T = 20°C, [GRB] = 19 μM, [D85E] = 21 μM, [C_D] = 20 μM, [C_E] = 38 μM, [DP_D] = 60 μM, [DP_E] = 40 μM, [KCl] = 150 mM, λ_M = 412 nm, λ_C = 465 nm, λ_{DP} = 485 nm.)

(detected by BHCCSE) proceeds 1.8 times more slowly in mutant than in wild-type, thus confirming the result obtained with fluorescein at pH 7.5. Although M rise is accelerated in wild-type BR at elevated pH, proton release is slightly slower. There might be a decelerating influence of the increased pH on the proton release reaction. But it should be mentioned that labelling with BHCCSE is not as selective as with FITC, although the same labelling conditions were applied.

M decay of the mutant is not affected by increasing the pH; however, proton uptake is slowed down by a factor of 3 as compared with pH 7.5. Proton uptake proceeds about 4 times more slowly in the mutant than in the wild-type and after M has completely decayed (Figures 2–4). Surface/bulk transfer of the pumped protons is not accurately monitored by BHCCSE. This is due to the general fact that the rate of any deprotonation reaction decreases with increasing pK of the dissociable group (Gutman, 1986). The detection of proton uptake by BHCCSE, however, is time-resolved. Using 1,3-dihydroxy-6,8-pyrenedisulfonate as pH indicator

for the bulk water phase confirms the above results. Proton uptake is slower in the mutant (DP_E in Figure 4) than in wild-type (DP_D). Detection of the surface/bulk proton transfer is not limited by the pK of DP because protonation reactions are diffusion controlled. From the data in Figure 4 (lowest panel), it seems that surface/bulk proton transfer is slower in the mutant than in wild-type. More measurements are required to clarify this point.

Additional experimental evidence for the delayed ejection of protons in mutant BR is obtained from measurements in which proton transfer processes in frozen membrane samples ($T = -15°C$) were detected with pyranine. We have previously shown that in ice the surface/bulk proton transfer is not rate-limiting for a pH indicator residing in the surrounding aqueous bulk phase, in contrast to the liquid state (Heberle and Dencher, 1990). Under these conditions the proton is detected in the Asp85 → Glu mutant after M has reached maximum concentration. Proton uptake proceeds after the absorbance changes at 412 nm have been completely relaxed (data not shown). Both findings are at variance with the wild-type. These measurements also refute the objection that the delayed appearance of protons at the membrane surface of mutant BR might be artificially induced by binding a pH indicator to the protein.

Discussion

Bacteriorhodopsin is a member of the family of 7-helix membrane spanning proteins, to which the visual pigment rhodopsin and many receptors also belong. Therefore, understanding the mechanism of BR function might also have important implications for the other members. One powerful approach towards unravelling the molecular steps of light-induced vectorial proton transfer is based on selective amino acid replacement in BR by site-specific mutagenesis. Although a wealth of information has recently been gathered employing mutated BR, which in part yielded quite useful insights into the pumping mechanism, some of the data are ambiguous. Especially experiments on amino acid substitutions obtained with *in vitro* mutated BR expressed in *Escherichia coli* are currently being repeated with mutated BR derived from the homologous system. As a result of the expression/reconstitution procedure applied, in *E.coli* expressed BR mutants as well as the expressed synthetic wild-type gene product exhibit pronounced structural and dramatic kinetic differences as compared with the respective proteins of halobacteria (Otto *et al.*, 1990; Marti *et al.*, 1991; Subramaniam *et al.*, 1991). Therefore, not even the data of the expressed 'wild-type' can be compared with the halobacterial wild-type. For at least four mutated BR species derived from halobacteria significant differences from the ones expressed in *E.coli* have been found (Bousche' *et al.*, 1992; Needleman *et al.*, 1991; Lanyi *et al.*, 1992; Zimanyi *et al.*, 1992). Additional alterations have to be considered that are not related to the specific amino acid replacement.

If, as in the present investigation, mutated BR is studied in its natural environment, i.e. in the crystalline hexagonal membrane, the data obtained can be interpreted in a more reliable manner. Furthermore, the crystalline membranes allowed characterization by X-ray diffraction with the conclusion that the replacement of aspartate by glutamate at position 85 does not result in alterations of the tertiary structure (at least not for the pump-active red chromophoric state; G.Büldt, N.A.Dencher and M.H.J.Koch, unpublished results). The changes in the kinetics of both the photocycle and the proton pumping cycle in the mutant investigated can therefore be solely attributed to functional differences in the exchanged amino acids.

According to the data presented here, replacement of Asp85 by Glu leads to a pronounced acceleration of M formation accompanied by retarded proton ejection to the extracellular membrane surface. This results in temporal decoupling of Schiff's base deprotonation on the one hand and proton transfer through the extracellular proton pathway on the other hand. Additionally, deceleration of proton reuptake is observed. The photocycle and the proton cycle of the red chromophoric state of the mutant, however, are basically the same as those of the wild-type. The M, N and O intermediates evolve (Figure 1) and the activation energies of photocycle and proton transfer steps (Figure 3A and B) are similar to those of the wild-type. However, a large transient accumulation of the O intermediate occurs. Any severe impairment of the structural and functional properties of BR due to the amino acid replacement can be excluded. Only by conservative amino acid exchange can useful information be gained about the involvement of an amino acid in the proton transport mechanism. Replacing Asp by Glu retains the property of large proton polarization, which is a requisite for a proposed proton transfer mechanism across BR (Olejnik *et al.*, 1992).

The observed acceleration of the M rise in the mutant (Figure 2), confirming previous studies (Butt *et al.*, 1989; Lanyi *et al.*, 1992), is indicative of a parallel increase in the rate of deprotonation of the Schiff's base. The faster Schiff's base deprotonation in the Asp85 → Glu mutant is a further hint that residue 85, perhaps together with Asp212 (Braiman *et al.*, 1988; Bousche' *et al.*, 1992), is the primary acceptor of the proton (Braiman *et al.*, 1988; Gerwert *et al.*, 1990; Metz *et al.*, 1992), possibly mediated by a water molecule (Dencher *et al.*, 1992). The observed acceleration of the internal transfer of the Schiff's base proton to the carboxylate of Glu might be explained by assuming that the additional methylenic group places the acceptor group closer to the donor (at maximum 1.3 Å, because the orientation of the side-chain is not known). This could facilitate formation of a hydrogen bond and light-induced proton transfer. Reducing the distance separating two groups will decrease the transition barrier for proton transfer and, consequently, also the transfer time (Scheiner and Duan, 1991). In the ground-state structure of wild type BR, on the other hand, the proton of the Schiff's base nitrogen possibly forms a hydrogen bond with Asp212. Asp85 might be too far away (Dencher *et al.*, 1992). It was proposed that during the BR_{568} to M_{412} transition, Asp85 and the Schiff's base transiently get closer, leading to the formation of a hydrogen bond and subsequent transfer of the proton to Asp85 (Dencher *et al.*, 1992). This transient approach is induced either by the observed movement of the Schiff's base linkage of the chromophore (see data discussed in Dencher *et al.*, 1992) and/or by the detected alterations in the tertiary structure of BR (Dencher *et al.*, 1989, 1991; Koch *et al.*, 1991). It is generally suggested (e.g. Marti *et al.*, 1991) that Asp85 is the counterion or belongs to the counterion complex of the retinylidene Schiff's base. Replacement of the Asp85 by Glu will result in a further stabilization of the protonated Schiff's base, i.e. increase in pK, if the carboxylate is

directed towards the Schiff's base. This, *per se*, would decrease the rate of deprotonation (Gutman, 1986), which is the opposite of what is observed in the mutant (Figure 2). But a decrease in the Schiff's base pK by 0.3 units was actually found for an Asp85 → Glu mutant. However, with the *E.coli* expression system utilized in these experiments, even the expressed 'wild-type' exhibits an apparent Schiff's base pK of only 11.3 (Marti *et al.*, 1991), as compared with 13.3 for BR in the purple membrane (Druckmann *et al.*, 1982). At present, the most plausible explanation for the accelerated deprotonation of the Schiff's base in the mutant is that the donor and acceptor group are closer together, reducing the transfer barrier. Even an alteration of only the relative orientation between the hydrogen bonding groups can lead to large alterations of the pK values and can shift the equilibrium position of the proton (Scheiner and Duan, 1991). However, it is the molecular structure of the active center during the L−M transition—and not of the ground state—that will determine the rate of proton transfer to the acceptor Asp85 or Glu85. In the M state, the environment of the retinal pocket (Moenne-Loccoz and Peticolas, 1992) and of Asp85 (Metz *et al.*, 1992) is more hydrophobic. This will stabilize the neutral pair configuration of the (deprotonated) Schiff's base and the (protonated) residue 85 (Scheiner and Duan, 1991). Obviously, in response to the light-induced alteration in the tertiary structure of BR (Dencher *et al.*, 1989, 1991; Koch *et al.*, 1991), the additional methylenic group of Glu85 might place the acceptor group in an environment of slightly different hydrophobicity from that sensed by Asp85.

Although M formation and deprotonation of the Schiff's base proceed much more rapidly in the mutant, proton ejection at the extracellular surface of BR does not follow this acceleration but is two orders of magnitude slower (Figure 2). This conclusion can only be drawn by the application of surface bound pH indicators. Indicators residing in the aqueous bulk phase are insufficient in this respect, since they monitor the delayed proton transfer from the membrane surface into the aqueous bulk. In contrast to wild-type BR, where M formation and ejection of protons occur concomitantly at neutral pH (Heberle and Dencher, 1990, 1992a,b), in the mutant the two processes are temporally decoupled. From these findings it follows that at least in the mutant but probably also in wild-type BR, deprotonation of the Schiff's base is not the rate-limiting step for the proton ejection cascade. Furthermore, since Asp85 remains protonated until the O intermediate (Siebert *et al.*, 1982; Gerwert *et al.*, 1990; Müller *et al.*, 1991; Bousche' *et al.*, 1992), the proton transferred from the Schiff's base to Asp85 is not the one appearing at the extracellular membrane surface in this reaction cycle. At least one additional group, XH, which could be an amino acid side-chain and/or a water molecule, is directly involved in the transfer process and is the transient source of the ejected proton. Additional protonatable groups, most likely water molecules (Papadopoulos *et al.*, 1990; Dencher *et al.*, 1992), participate in the proton release pathway. It is often suggested that Arg82 is the proton release group, XH. Upon deprotonation of the Schiff's base and protonation of Asp85, the salt bridge between Asp85 and Arg82 is broken. The protonation of Asp85 lowers the pK of Arg82 by destabilizing arginine's positive charge, and could result in deprotonation of Arg82 and ejection of the proton. However, since the results reported on in *E.coli*-expressed Arg82 mutants are highly controversial and because the pK_a of XH becomes ~5.8 in M (Zimanyi *et al.*, 1992), Arg82 probably has to be replaced by either H_3O^+ or another amino acid residue in the scenario described above.

In wild-type BR, the Schiff's base proton protonates Asp85 and via a 'domino effect' a proton is ejected from XH almost simultaneously to the extracellular surface of BR (Dencher *et al.*, 1992; Heberle and Dencher, 1992b). In the mutant Asp85 → Glu, on the contrary, there is a great delay between the former and latter steps. This indicates that a reaction involved in deprotonation of XH, triggered by the protonation of Asp85, is the rate-limiting step for proton ejection. The changes in the tertiary structure of BR might be the trigger for this event (Dencher *et al.*, 1989, 1991; Koch *et al.*, 1991). The observed 1.8-fold retardation of the proton inside the extracellular pathway in the mutant, i.e. ejection after 157 instead of 92 μs (Table I), is in line with the suggested shorter distance and/or more optimal spatial arrangement between the Schiff's base and Glu85, since this inherently leads to a less favorable interaction between Glu85 and XH. Remarkably, the ratio of the rates of proton ejection in mutant and wild-type BR is similar to the ratio of the dissociation constants of Asp and Glu.

The Asp85 → Glu substitution also affects the proton reuptake reaction at the cytoplasmic side, although residue 85 is located in the proton release pathway. Proton uptake is 4- to 5-fold slower in the mutant than in the wild-type and occurs during the lifetime of the O intermediate. We propose that there is a feedback mechanism connecting the protonation states of Asp85 and Asp96 in the proton release and uptake pathway, respectively. This is supported by the observation that replacement of Asp96 by Asn not only dramatically decelerates M decay, but also slows down its formation 2- to 3-fold (Heberle, 1991; Zimanyi *et al.*, 1992).

It is worth mentioning that neither the slowed proton ejection nor the delayed proton uptake in the Asp85 → Glu mutant are accelerated by the addition of azide (J.Heberle and N.A.Dencher, unpublished results), while both are restored in an Asp96 → Asn mutant (Tittor *et al.*, 1989).

Asp85 plays an essential role in the proton release reaction from the Schiff's base to the extracellular membrane surface and in the subsequent proton uptake at the cytoplasmic surface. The importance of adequate distances between the Schiff's base proton and the primary proton acceptor, as well as between the primary and the secondary proton acceptor(s)/donor(s), was unequivocally demonstrated by the Asp85 → Glu mutant. The spatial orientation will determine the pK values of these groups by positioning them in an adequate hydrophilic/hydrophobic environment and at an appropriate distance from charged residues.

Materials and methods

The Asp85 → Glu point mutant of BR within the natural environment of the PM was obtained by mutagenesis of the wild-type GRB strain and the bacterioopsin gene was sequenced as described previously (Soppa and Oesterhelt, 1989). For the proton transfer experiments, derivatives of the pH indicators fluorescein [fluorescein-5-isothiocyanate (FITC), Molecular Probes, Eugene, OR] or of coumarin [3-(2-benzothiazolyl)-7-hydroxycoumarin-4-carboxylic acid succinimidyl ester (BHCCSE), Lambda Probes, Graz, Austria (Fauler, 1989)] were covalently conjugated to Lys129 at the extracellular surface of BR (wild-type and mutated, respectively). Unbound pH indicator was removed by washing the labelled PM three times

in 0.1 M $NaHCO_3$ (pH 8.5) and thereafter incubating overnight at 4°C before carrying out three additional washes in 150 mM KCl [for a detailed labelling protocol refer to Heberle and Dencher (1990, 1992a,b)]. The molar ratio of bound pH indicator to BR was 0.5–0.7 for FITC and 1.5–2.5 for the coumarin derivative. Whereas FITC does not change its spectroscopic properties upon binding, the coumarin derivative exhibits a 20 nm redshift of its absorption spectrum and a pK shift of 1.5 pH units into the alkaline region (pK = 8.5, Heberle, 1991). As indicator of pH changes in the bulk water phase at neutral pH values, pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate, Kodak, pK = 7.2) (Grzesiek and Dencher, 1986) was used. A derivative of pyranine, 1,3-dihydroxy-6,8-pyrenedisulfonate, (Lambda Probes, Graz, Austria) with two pKs (pK_1 = 7.1, pK_2 = 8.65) served to detect pH changes in the range of 7–9 (Wolfbeis *et al.*, 1983).

Transient absorption spectroscopy was performed by employing a frequency-doubled Nd:YAG laser (λ = 532 nm, E = 7 mJ/pulse/cm^2, repetition frequency <0.5 Hz, Spectra Physics, Darmstadt, Germany) to excite the samples. Control experiments with lower excitation energy ruled out any deleterious effect on the samples. Changes in absorbance at selected wavelengths were digitized with a transient recorder (eight different sampling rates, 100 MHz maximum sampling rate, 10 bit resolution, 512 kwords memory, Dr Strauss, Gundelsheim, Germany). This set-up enabled us to detect transients over at least 8 decades in time with a resolution of 20 ns. The high density of sampling points leads to a high signal-to-noise ratio (S/N) when neighboring points are co-added. Further improvement of the S/N is achieved by averaging 100–200 data traces. Details of the transient absorption spectrometer are described by Thiedemann *et al.* (1992). Fitting of the weighted data points to a sum of exponentials was done with a program that uses the Marquardt–Levenberg algorithm. The fit was accepted when the residuals were free from systematic deviations.

When applying pH indicators to detect proton migration, absorbance changes due to the photocycle have to be considered. In addition, surface bound pH indicators detect transient modulations of the membrane surface potential (Heberle and Dencher, 1992b). Both contributions are eliminated by subtracting a measurement of a sample without buffer and the same sample in a highly buffered medium (10 mM imidazol or Tris). The buffers do not affect the photocycle kinetics. The difference leads to the pure protonic response of the pH indicator.

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