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Substitution of amino acids Asp-85, Asp-212, and Arg-82 in bacteriorhodopsin affects the proton release phase of the pump and the pK of the Schiff base

(site-specific mutagenesis/purple membrane/proton pump/photocycle/photovoltage)

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ABSTRACT **Photocycle and flash-induced proton release and uptake were investigated for bacteriorhodopsin mutants in which Asp-85 was replaced by Ala, Asn, or Glu; Asp-212 was replaced by Asn or Glu; Asp-115 was replaced by Ala, Asn, or Glu; Asp-96 was replaced by Ala, Asn, or Glu; and Arg-82 was replaced by Ala or Gln in dimyristoylphosphatidylcholine/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate micelles at pH 7.3. In the Asp-85 → Ala and Asp-85 → Asn mutants, the absence of the charged carboxyl group leads to a blue chromophore at 600 and 595 nm, respectively, and lowers the pK of the Schiff base deprotonation to 8.2 and 7, respectively, suggesting a role for Asp-85 as counterion to the Schiff base. The early part of the photocycles of the Asp-85 → Ala and Asp-85 → Asn mutants is strongly perturbed; the formation of a weak M-like intermediate is slowed down about 100-fold over wild type. In both mutants, proton release is also slower but clearly precedes the rise of M. The amplitude of the early (<0.2 μs) reversed photovoltage component in the Asp-85 → Asn mutant is very large, and the net charge displacement is close to zero, indicating proton release and uptake on the cytoplasmic side of the membrane. The data suggest an obligatory role for Asp-85 in the efficient deprotonation of the Schiff base and in the proton release phase, probably as proton acceptor. In the Asp-212 → Asn mutant, the rise of the absorbance change at 410 nm is slowed down to 220 μs, its amplitude is small, and the release of protons is delayed to 1.9 ms. The absorbance changes at 650 nm indicate perturbations in the early time range with a slow K intermediate. Thus Asp-212 also participates in the early events of charge translocation and deprotonation of the Schiff base. In the Arg-82 → Gln mutant, no net transient proton release was observed, whereas, in the Arg-82 → Ala mutant, uptake and release were reversed. The pK shift of the purple-to-blue transition in the Asp-85 → Glu, Arg-82 → Ala, and Arg-82 → Gln mutants and the similarity in the photocycle and photoelectrical signals of the Asp-85 → Ala, Asp-85 → Asn, and Asp-212 → Asn mutants suggest the interaction between Asp-85, Arg-82, Asp-212, and the Schiff base as essential for proton release.**

Site-directed mutagenesis has shown Asp-96, Asp-85, and Asp-212 to be essential for proton translocation by bacteriorhodopsin (bR) (1). The very low activity in mutants Asp-96 → Ala (D96A) and Asp-96 → Asn (D96N) at pH 7 is due to a markedly slowed-down decay of the photocycle intermediate M and the associated charge movement (2–5). Asp-96 was concluded to be the internal proton donor for the reprotonation of the Schiff base (SB) leading to the decay of M (3, 4). Fourier-transform infrared spectroscopy has revealed the protonation states of Asp-85, Asp-96, Asp-115, and Asp-212 in the K, L, and M intermediates and provided clues to the time course of proton transfer (6, 7). The data suggest that Asp-85 and Asp-212 are deprotonated in the ground state and become protonated in the L → M transition (6). About 30% of the internal charge displacement is associated with the L → M transition (10–100 μs) (8), and proton release is detected in the aqueous solution on the extracellular side of the membrane in this time range (9). To further investigate the H^+ release step, we have studied the time course of the photocycle, photovoltage, and release and subsequent reuptake of protons in single-substitution mutants of amino acids Asp-85, Asp-96, Asp-115, Asp-212, and Arg-82. Our results show that Asp-85, Asp-212, and Arg-82 are involved in the proton release phase and suggest that Asp-85 serves as acceptor of the proton from the protonated SB. In the ground state, Asp-85 probably serves as counterion to the SB; its substitution by neutral amino acids causes red shifts in the absorption maximum and lowers the pK of the SB deprotonation.

MATERIALS AND METHODS

The construction of the genes coding for the Asp → Asn, Asp → Glu, and Arg → Gln mutants, their expression in *Escherichia coli*, the purification of the bacterioopsin apoproteins, and the regeneration with retinal in 1% dimyristoylphosphatidylcholine (DMPC)/1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) micelles have all been described (1, 10). The preparation of the Asp → Ala and Arg → Ala mutants was by identical procedures. The mutant apoproteins were also regenerated and reconstituted in vesicles with the polar lipids from *Halobacterium halobium* in a 1:1 weight ratio (HL vesicles) (11).

For flash photolysis and pH indicator experiments, the micelles were dialyzed against a solution containing 1% DMPC, 1% CHAPS, 150 mM KCl (no buffer) at pH 7.3. All mutants were purple, except for D85A and D85N, which were blue (see Table 1). In 1% DMPC, 1% CHAPS, 150 mM

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Abbreviations: bR, bacteriorhodopsin; ebR, bacteriorhodopsin prepared from the expression of a synthetic wild-type gene in *Escherichia coli*; DMPC, dimyristoylphosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HL vesicles, bR or its mutants reconstituted in vesicles consisting of the polar lipids of *Halobacterium halobium* in a 1:1 weight ratio; SB, unprotonated Schiff base. bR mutants are designated by the wild-type amino acid residue (single-letter code) and its position number followed by the substituted amino acid residue. For example, in mutant D85N, Asp-85 is replaced by Asn.

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KCl, 50 mM sodium phosphate buffer, the mutants D85E, R82A, and R82Q have a purple-to-blue transition with pK values near neutral (6.4, 6.4, and 7.0, respectively; see refs. 1, 5, 10, and 12), and D212E has a blue-to-pink (510 nm) transition with a pK of 8.0 (12).

The photocycle was measured with a homemade flash photolysis spectrometer (4).

Photovoltage was measured on HL vesicles adsorbed to black lipid membranes as described (3, 8).

The pH changes associated with proton release and uptake were measured at pH 7.3 by using the indicator dye pyranine (8-hydroxyl-1,3,6-pyrenetrisulfonate; pK = 7.2) as described (3, 9). The stoichiometry of protons released per bR cycling was calculated from the measured absorbance difference due to pyranine ($\Delta\Delta A_{450}$), the calibration of the absorbance at 450 nm in terms of protons ($\Delta A_{450}/\Delta[H^+]$), the extinction coefficient ($\varepsilon_{570/410}$) of bR or M, the absorbance change at 570 or 410 nm ($\Delta A_{570/410}$), and the length of the cuvette d as follows:

$$n_{H^+} = \left[\frac{\Delta\Delta A_{450}}{\Delta A_{450}/\Delta[H^+]}\right] \cdot \left[\frac{\varepsilon_{570/410} \cdot d}{\Delta A_{570/410}}\right].$$

The inverse of the second factor is the concentration of bR that has entered the photocycle.

RESULTS

The time course of the photocycle at 650 and 410 nm as well as the kinetics of proton release and uptake, as indicated by the dye pyranine, were measured. The data for bR prepared from the expression of a synthetic wild-type gene in *E. coli* (ebR) and five selected mutants are shown in Fig. 1. The corresponding kinetic parameters and stoichiometries of proton pumping are summarized in Table 1 for all samples examined.

ebR. bR in DMPC/CHAPS micelles at pH 7.3 exhibits an accelerated rise and a slow, biphasic decay of the M intermediate, as compared to purple membrane [but is quite similar to purple membrane at pH 9 (4, 13)]. The proton release time is probably faster than the observed 110 μs (Fig. 1*A Bottom*), representing the response time of the method. The biphasic nature of the M decay is also apparent in the proton uptake. From the amplitude of the dye signal, a stoichiometry of 0.8 ± 0.2 proton per bR cycling was obtained. The complete photocycle of ebR was measured from 370 to 690 nm in steps of 20 nm. This is illustrated in Fig. 2*A*, which shows the projection of the $\Delta A(\lambda, t)$ surface on the (λ, t) plane.

D85 Mutants. Asp-85 is believed to be deprotonated in the ground state and to become protonated in the L → M transition (6). The proton-pumping activity of D85E in the purple state is ≈30–40% of wild type (1, 12). In this form, the mutant has a proton release stoichiometry close to 1 and a rise of M that is accelerated to 2.6 μs (Table 1). The mutants D85N (1) and D85A, which lack the carboxyl group, are completely inactive in proton pumping, are blue (λ_{max} = 595 and 600 nm, respectively), and do not turn purple at any pH. In D85N, with increasing pH, the absorbance around 600 nm decreases, and a species at 405 nm is formed in a reversible way (Fig. 3*A*). In Fig. 3*B*, the titration data are fitted according to a simple equilibrium $AH_n \rightleftharpoons A + nH$, yielding a pK of 7.0 and n = 0.85. For D85A, the corresponding numbers are pK = 8.2 and n = 0.90 (data not shown). Apart from a 5-nm overall blue shift, the difference spectrum of this species (absorption maximum at 405 nm and shoulders at 380 and 425 nm) has the characteristic features of the M state of the photocycle (14), indicating that the SB is deprotonated.

In D85A, the rise of the flash-induced absorbance change around 410 nm is more than 100 times slower than that in wild type (2.8 ms, Fig. 1*B*). Fig. 2*B* shows a delayed but well-defined M-like intermediate of small amplitude. An analysis of the amplitude spectra obtained from multiwavelength fits supports this conclusion. Comparison of Fig. 1*B Middle* with Fig. 1*B Bottom* shows that the release of protons (620 μs) is also slowed down but precedes the rise of M (2.8 ms). This is also the case for D85N (490 μs and 1.4 ms; see Table 1). The apparent stoichiometries of proton pumping for D85N and

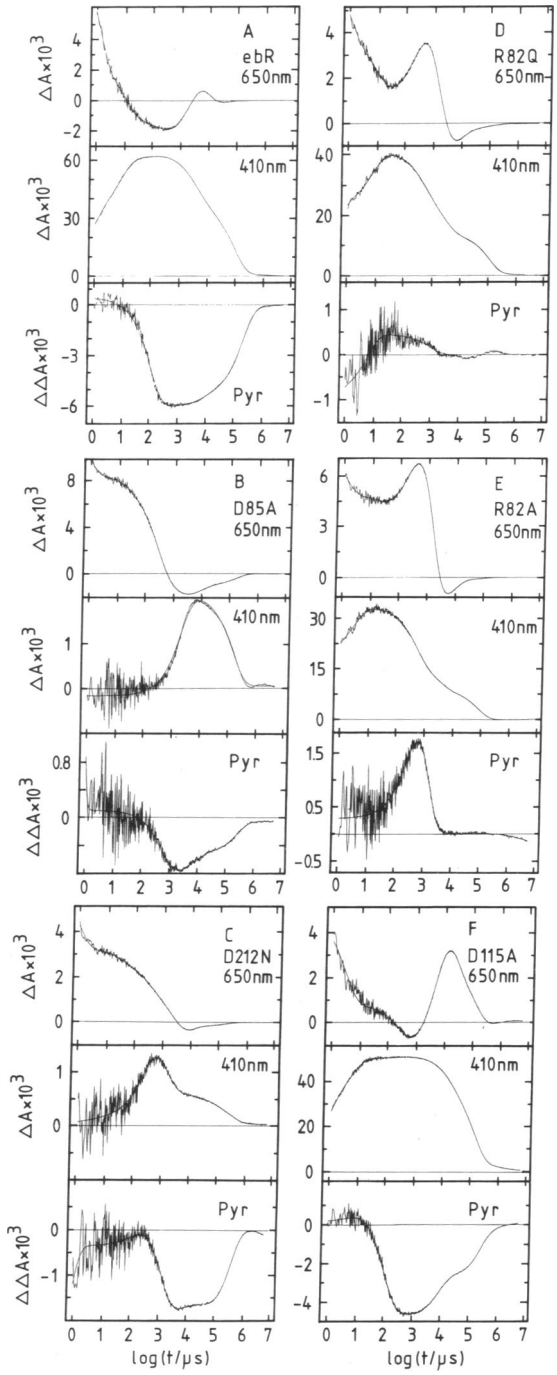

FIG. 1. Time course of the absorbance changes at 650 nm (*Top*) and 410 nm (*Middle*) during the photocycle and at 450 nm for the pH indicator pyranine (Pyr) (*Bottom*) for various mutants of bR in 1% DMPC/1% CHAPS micelles at pH 7.3. (*A*) ebR. (*B*) D85A. (*C*) D212N. (*D*) R82Q. (*E*) R82A. (*F*) D115A. The logarithmic time scale is from 1 to 10^7 μs in each panel. A negative $\Delta\Delta A$ indicates the release of protons. Solid lines are multiexponential fits. Conditions were 150 mM KCl at pH 7.3 (unbuffered), 22°C, dark-adapted; excitation was at 590 nm (3 mJ, 10 ns); the repetition rate was less than 0.1 s^{-1}.

Table 1. Kinetics of rise and decay of M, kinetics of proton release and uptake, and stoichiometry of proton pumping

		M kinetics†		H^+ kinetics†		
Protein	λ_{max}*	Rise, μs	Decay, ms	Release, μs	Uptake, ms	n_{H^+}‡
ebR	550	<1.3/13	6/170	110	9/160	0.8
D85A	600	2800	230	620	8/300	1.2
D85N	595	1400	100	490	10/110	1.9
D85E	543	2.6	7/350	150	6/640	0.8
D96A	558	5/39	7000	90	7000	0.9
D96N	558	3/200	300/10000	100	150/5000	1.3
D96E	552	3/30	4/900	120	1.5/800	1.0
D115A	543	<1/5	15/130	110	7/340	0.8
D115N	538	5/70	12/300	180	10/500	1.3
D115E	538	4/130	20/250	120	7/300	0.9
D212E	560	2.0	0.6/850	570	24/1100	1–2
D212N	555	220	1.6/200	1900	340	2–4
R82A	547	<0.2/3	0.7/60	1000	0.44	1
R82Q	547	0.4/7	2/100	—	—	0

Data was taken with 1% DMPC/1% CHAPS micelles at pH 7.3 in 150 mM KCl (no buffer).
*Wavelength of the maximum absorption in nm in the dark-adapted state.
†When a rise or decay is multiphasic, the two main times are given.
‡Number of protons released and taken up per bR cycle.

D85A are 1.9 and 1.2, respectively, and appear to be too large. The absorbance change at 650 nm (D85A; Fig. 1*B Top*) is unusually large and contains a major component at 500 μs that correlates with the proton release time. Fig. 2 *A* and *B* illustrate the substantial differences between the photocycles of ebR and D85A.

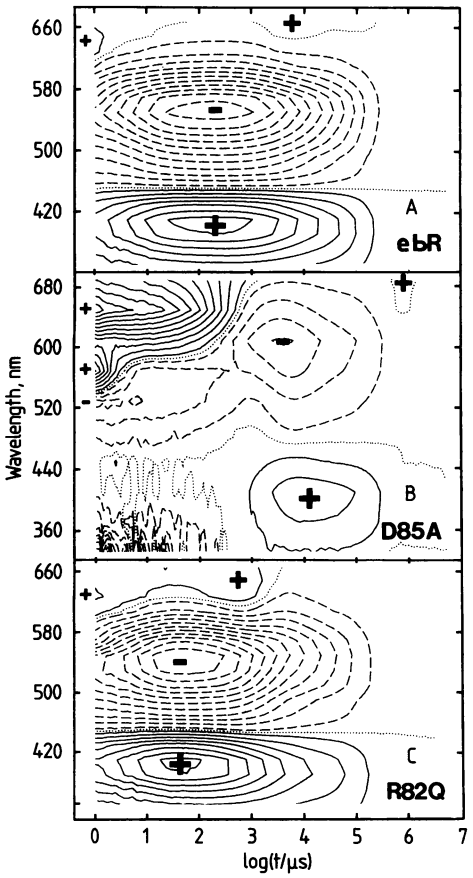

FIG. 2. Contour plots in the (λ, t) plane for the absorbance changes [$\Delta A(\lambda, t)$] of ebR (*A*), D85A (*B*), and R82Q (*C*). Lines of equal ΔA are plotted, and maxima and minima are labeled with + and −, respectively. The photocycle was measured in steps of 20 nm from 350 to 670 nm for ebR and R82Q and in steps of 40 nm from 330 to 690 nm for D85A. Conditions were as in Fig. 1.

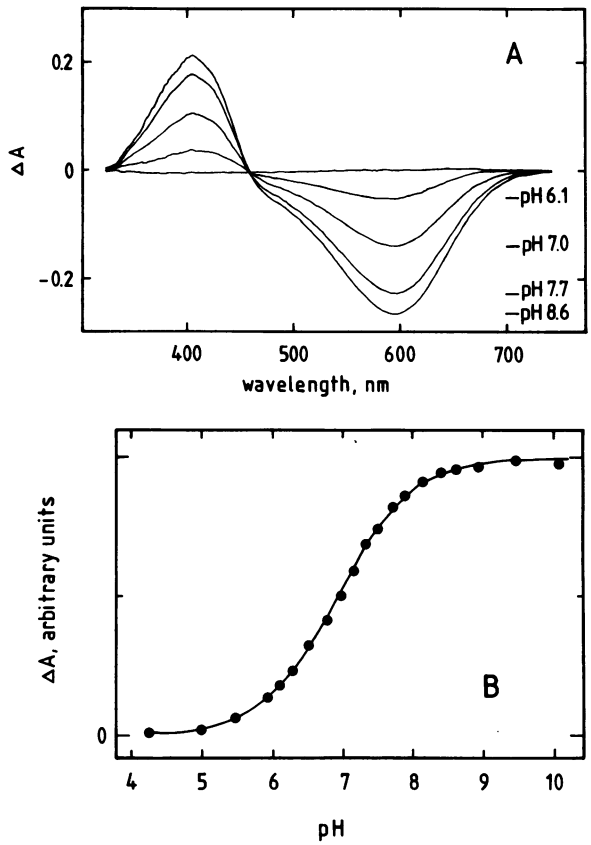

FIG. 3. (*A*) Titration of the blue mutant D85N. Difference spectra of D85N at various pH values against D85N at pH 4.2. At pH 4.2, the spectrum of D85N has no contribution around 400 nm. Spectra were recorded at 18 pH values. Only the spectra at pH 6.1, 7.0, 7.7, and 8.6 are shown. The conditions were 1% DMPC/1% CHAPS micelles in 150 mM KCl/30 mM sodium phosphate buffer. (*B*) Amplitude of the spectral titration data at 600 nm from *A* plotted versus pH. The fit corresponds to a pK of 7 and a stoichiometry of 0.85 protons.

In Fig. 4, the time-resolved photovoltage for D85N is compared with ebR. A negative signal indicates proton displacement in the direction of wild-type pumping. The electrical processes preceding the decay of M are strongly perturbed in D85N. The unresolved fast charge translocation

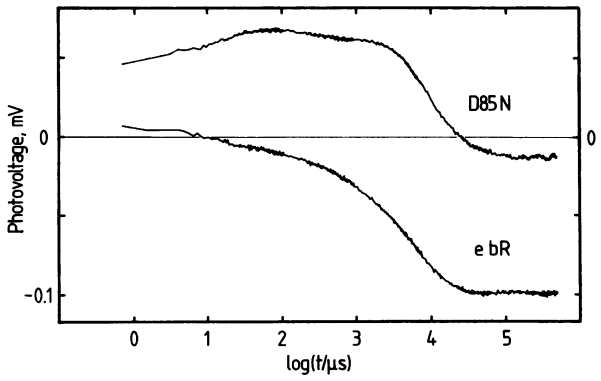

FIG. 4. Time course of the photovoltage in HL vesicles of ebR and D85N. The common base line gives the pretrigger level. The passive system discharge ($\tau \approx 2$ s) is removed by deconvolution (8). The conditions were 150 mM KCl/30 mM sodium phosphate buffer at pH 6, 25°C, excitation at 575 nm (45 μJ, 10 ns), ebR on Teflon support, and D85N on black lipid membrane support.

in the direction opposite to that of the net transport is much larger than in wild type, and a charge movement in the reverse direction is also apparent in the 10- to 100-μs range. The photovoltage returns almost to the original level with a time constant of ≈10 ms, which is close to normal for the reprotonation of the SB from the cytoplasm.

D212 Mutants. The photocycle and proton release also differ substantially in D212N from wild type (Fig. 1*C*). The amplitude of the absorbance change at 410 nm is very small, and its rise (220 μs) is markedly delayed. The decay is biphasic, with a fast component at 1.6 ms and a slow component at 200 ms. As in D85N, the photocycle is severely perturbed in the D212N mutant. At 650 nm, the absorbance changes for D85N and D212N are remarkably similar. The proton release time is slowed down to 1.9 ms and correlates with the fast 410-nm decay and with a 2-ms component in the decay at 650 nm (Fig. 1*C Top*). These results indicate that the rapid decay phase of the 410-nm signal in this mutant is not due to a real M-like intermediate, and more detailed experiments elucidating the nature of the intermediates are required. The calculated stoichiometry of proton pumping in D212N is between 2 and 4, which is very different from the usual values, suggesting erroneous assumptions for the stoichiometry calculation due to markedly different intermediates. In the D212E mutant, the rise time of M is 2 μs, which resembles the effect of D85E (Table 1). However, the release of protons is as slow as 570 μs. The decay of M, the depletion signal, and the proton uptake are all slowed down to ≈1 sec. Since the stoichiometry of proton pumping is 1, the observed reduced proton-pumping activity of this mutant is probably due to the slow turnover of the pump as in D96N and D96A.

R82 Mutants. The two mutants R82Q and R82A have similar photocycle kinetics as shown in Figs. 1*D*, 2*C*, and 1*E*. Both mutants have a normal M amplitude, with rise and decay times that are faster than in wild type. Fig. 2*C* shows that the photocycle of R82Q mainly differs from wild type in the region above 620 nm. Fig. 1*D* (*Top*) and Fig. 1*E* (*Top*) illustrate that the absorbance signals at 650 nm are changed in the two mutants R82A and R82Q; R82Q shows a peak with rise and decay times of 300 μs and 1.4 ms, respectively. This may be L or an early O intermediate. For R82Q, the dye signal amplitude is very small, consistent with an apparent stoichiometry of 0 (Fig. 1*D*). For R82A (Fig. 1*E*), a positive spike-like ΔpH signal is observed with reversed sign, suggesting that in this mutant proton uptake precedes release. The rise and decay times (440 μs and 1 ms) correspond closely to those of the positive peak at 650 nm. The dye signal of Fig. 1*E* disappeared in the presence of 10 mM phosphate buffer, proving that it is indeed due to transiently released protons. The photovoltage signals of R82A and R82Q in the purple state show a markedly reduced amplitude and acceleration of the microsecond component (data not shown), which is believed to reflect the proton release step.

D115 Mutants. In D115A, D115N, and D115E, the main differences with wild type are a slower M decay and H^+-uptake time and a reduced decay rate of O (leading to a larger amplitude of this intermediate at 650 nm, Fig. 1*F*).

D96 Mutants. In D96A and D96N, the decay of M and the associated proton uptake is dramatically slowed down to 7 s without effect on the rise of M, the proton release time, and the stoichiometry (0.95) (Table 1; data shown in ref. 4). These results support the previous conclusion that the low activity in these mutants (2–3% at pH 7) is due to the slow turnover of the pump and that Asp-96 serves as the internal proton donor for the reprotonation of the SB (3, 4). The decay of M in the D96E mutant, on the other hand, is much faster than in D96N or D96A, indicating that Glu can apparently replace Asp as the internal donor. The results obtained in micelles thus closely resemble those previously obtained in HL vesicles (3, 4).

DISCUSSION

The functional cycle of bR consists of two major electrogenic steps. In the first microsecond phase, the SB is deprotonated, leading to the formation of M and to the release of a proton on the extracellular side of the membrane. In the second millisecond stage, the SB is reprotonated, M decays, and a proton is taken up from the cytoplasm. Substitution of Asp-96 and Asp-115 only affects the second half of this cycle (2–5). Asp-96 remains protonated until the decay of M (7) and serves as the internal proton donor in the reprotonation of the SB (3, 4). Asp-115 remains protonated during the entire cycle (6), and mutations of this amino acid lead mainly to a slowdown in the decay of O. Whereas the role of Asp-96 in the SB reprotonation has been clarified, the amino acids mediating the proton release from the SB and the acceptor of this proton have not been identified. This has been the major focus of the present work. The results described above show that Asp-85, Asp-212, and Arg-82 are involved in this process. Substitution of these residues affects the early part of the photocycle, the proton release, and the pK of the purple-to-blue transition. Asp-85 and Asp-212 are probably deprotonated in the ground state (6), and Arg-82 is presumably positively charged. These charged residues may interact with the protonated SB and with each other. Specifically the negative charge of Asp-85 is probably in close proximity to the positively charged guanidinium group of Arg-82, which keeps Asp-85 in the ionized form and lowers its pK. We postulate that when Asp-85 is ionized the chromophore absorbs around 550 nm (in micelles), but, when it is protonated or replaced by a neutral amino acid, the absorbance is red-shifted to between 585 and 605 nm. A number of experimental results support this view. In wild-type bR, the interaction between Asp-85 and Arg-82 leads to an apparent pK of 3 for the purple-to-blue transition. In D85E, the pK is shifted to 6.4 (12), probably due to the change in position of the carboxyl group. In R82A and R82Q, the absence of the positive charge results in a shift of the pK of the color transition to 6.4 and 7.0, respectively. These results indicate that the purple-to-blue transition is controlled by the protonation state of Asp-85 (10, 12). This conclusion receives strong support from the present experiments. Thus, substitution of Asp-85 with the neutral amino acids Ala or Asn results in a blue chromophore that does not convert to a purple form: no negative charge can be generated at position 85 by increasing the pH. We note that the spectral red shift upon removal of the negative charge at Asp-85 is in accordance with the idea that Asp-85 serves as counterion to the protonated SB in wild-type bR.

At alkaline pH, a yellow species (405 nm) is formed in D85A and D85N with an absorption spectrum characteristic of a deprotonated SB linkage. Thus, a neutral residue at position 85 shifts the pK for SB deprotonation in the dark from ≈13 (15, 16) for wild type to between 7 and 8. These values are close to those reported for SB model compounds in solution (16). The effect of the charge at position 85 on the pK of the SB requires that this residue interacts with and is close to the protonated SB. The effect of the neutral mutants D85A and D85N on the pK of the SB deprotonation and the role of Asp-85 in the purple-to-blue transition suggest that Asp-85 may be the primary counterion to the protonated SB. ^{15}N NMR experiments on the SB nitrogen indicate that the counterion is complex, involving several amino acids and water, and lead to the interpretation of the purple-to-blue transition in wild type as the protonation of the complex counterion (ref. 17, also see refs. 18 and 19). There is substantial evidence (1) that Asp-212 also interacts with the chromophore. Asp-212 may be part of a weak complex counterion (18) and may interact with the Asp-85/Arg-82 ion pair. It is likely that Asp-212 is deprotonated in wild type (6)

and in D85A and D85N. The red shift in D85A and D85N, the lack of a red shift in D212N and D212A at neutral pH, and the absence of a decrease in the pK of the SB deprotonation in D212N indicate that the carboxyl group of Asp-212 is not as close to the SB as Asp-85 or may be in an unfavorable orientation for interaction. Asp-212 may function as counterion when the negative charge at Asp-85 has been removed (12).

In the D85A, D85N, and D212N mutants, the early phase of the photocycle is strongly perturbed. In D85A and D85N, the formation of M is delayed (100-fold compared to wild type), and the amount of M is small. Asp-85 has been proposed as the primary acceptor of the SB proton on the basis of Fourier-transform IR measurements, indicating protonation of Asp-85 in the L → M transition (6). One might therefore expect to observe no M intermediate in the D85A and D85N mutants. Nevertheless, a greatly delayed M of small amplitude was observed, indicating that its formation is not completely blocked (Figs. 1*B* and 2*B*). In HL vesicles of D85N, no M was detected at pH 6, whereas a minor amount was present at pH 8 (20). This is in agreement with our results with micelles at pH 7.3, since the photocycle of ebR in micelles at this pH is equivalent to that of HL vesicles around pH 9 (see *Results*). We believe that in the D85A and D85N mutants the normal formation of M is blocked and that the appearance of a minor amount of much delayed M is due to other mechanisms. At sufficiently high pH, deprotonation of the SB could occur, for instance, directly to the external medium. Alternatively, at these pH values close to the pK of SB deprotonation (7, 8), flash excitation may lower the pK and shift the equilibrium transiently toward the deprotonated SB. We note that there is no contradiction with the absence of a well-defined M intermediate in the blue membrane of wild type (21), since this blue form only exists at low pH. At 650 nm, the photocycle kinetics are changed in D85A and D212N in a strikingly similar way (Fig. 1 *B* and *C*), indicating that these mutations in helices C and G, respectively, lead to the same perturbation. The retardation in the release of protons suggests that both aspartate residues are involved in mediating the release of protons to the extracellular side and are essential for a normal first half of the photocycle. The photovoltage results on the blue mutant D85N suggest that no net proton transport occurs and that release and uptake occur on the same side of the membrane. Assuming that the protein orientation in HL vesicles of wild type and D85N is the same, the protons are released to the cytoplasmic side in D85N. This finding explains why no pumping activity was detected in this mutant (1) and why the mutant D85E in the blue form is inactive as well (5, 12). The acid blue form of wild type shows photovoltage signals (22) that are similar to those detected for D85N (Fig. 4). The fact that proton release precedes the rise of M in D85N and D85A further suggests that the released proton is not the SB proton, at least in these mutants. Taken together, our data indicate that Asp-85 is a good candidate for acceptor of the SB proton.

In mutants R82Q and R82A, an M intermediate is formed in normal amounts. In the range of 650 nm, the photocycle is perturbed both in the early (K and L intermediates) and in the later part (≈1 ms). Although both mutants are normally active in proton pumping in the purple form (10, 12), a transient pH change is not detectable in R82Q and is reversed in sign in R82A. In R82A, the release and uptake times are quite similar, and it takes only a small change in one of them to reduce the net proton release signal to zero, as in R82Q. These experiments indicate that the proton release is strongly retarded. Electrical data and the fast rise of M suggest that the SB is rapidly deprotonated in these mutants. This proton may get trapped within the protein, possibly between Asp-85 and Asp-212 when the positive charge of Arg-82 is absent. Proton uptake and reprotonation of the SB appear to be required for the liberation of a proton to the extracellular side.

In conclusion, the data presented above on the Asp-85, Asp-212, and Arg-82 mutants show that all three amino acids are involved in the SB deprotonation and proton release mechanism in the early phase of the bR photocycle.

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