Perturbed Interaction between Residues 85 and 204 in Tyr-185→Phe and Asp-85→Glu Bacteriorhodopsins

Hans-Thomas Richter,* Richard Needleman,# and Janos K. Lanyi*
*Department of Physiology and Biophysics, University of California, Irvine, California 92717, and #Department of Biochemistry, Wayne State University, Detroit, Michigan 48201 USA

ABSTRACT According to earlier reports, residue 85 in the bacteriorhodopsin mutants D85E and Y185F deprotonates with two apparent pK_a values. Additionally, in Y185F, Asp-85 becomes significantly more protonated during light adaptation. We provide a new explanation for these findings. It is based on the scheme that links the protonation state of residue 85 to the protonation state of residue 204 (S.P. Balashov, E.S. Imasheva, R. Govindjee, and T.G. Ebrey. 1996. *Biophys. J.* 70:473–481; H.T. Richter, L.S. Brown, R. Needleman, and J.K. Lanyi. 1996. *Biochemistry.* 35:4054–4062) and justified by the observation that the biphasic titration curves of D85E and Y185F are converted to monophasic when the E204Q residue change is introduced as a second mutation. Accordingly, the D85E and Y185F mutations are not the cause of the biphasic titration, as that is a property of the wild-type protein. By perturbing the extracellular region of the protein, the mutations increase the pK_a of residue 85. This increases the amplitude of the second titration component and makes the biphasic character of the curves more obvious. Likewise, a small rise in the pK_a of Asp-85 when the retinal isomerizes from 13-*cis*,15-*syn* to all-*trans* accounts for the changed titration behavior of Y185F after light adaptation. This mechanism simplifies and unites the interpretation of what had appeared to be complex and unrelated phenomena.

INTRODUCTION

The anionic state of Asp-85 has two consequences in the light-driven proton pump bacteriorhodopsin. First, the negative charge of Asp-85 is the main component of the counterion to the positively charged protonated retinal Schiff base (Subramaniam et al., 1990, 1992; Marti et al., 1991; Greenhalgh et al., 1992; Brown et al., 1993), and thereby it keeps the absorption maximum of the chromophore near 570 nm rather than at longer wavelengths. Second, after photoisomerization of the retinal, Asp-85 is the acceptor in the deprotonation of the Schiff base, the first proton transfer step in the photochemical cycle (Braiman et al., 1988; Otto et al., 1990; Subramaniam et al., 1992; Metz et al., 1992). A low pK_a for Asp-85 ensures that it is anionic and can fulfill these functions under physiological conditions. Indeed, in the unphotolyzed wild-type protein, this pK_a is approximately 2.5 (Fischer and Oesterhelt, 1979; Mowery et al., 1979; Subramaniam et al., 1990; Jonas and Ebrey, 1991). Below this pH the chromophore changes reversibly from purple (maximum at 568 nm) to blue (maximum at 603 nm), the result of removing the negative charge of Asp-85. Lacking a proton acceptor in the blue form, the Schiff base does not deprotonate upon photoexcitation (Mowery et al., 1979; Váró and Lanyi, 1989).

There are examples, however, when the titration of residue 85 gives different or more complex results. In the R82Q mutant, the blue-to-purple transition occurs near pH 7 (Stern and Khorana, 1989; Otto et al., 1990; Miercke et al., 1991; Balashov et al., 1993; Brown et al., 1993), an effect attributed to having removed the electrostatic stabilization from the anionic Asp-85 that lowered its pK_a. In the D85E mutant, the titration is biphasic, with pK_a values at approximately 4 and 9 for the blue-to-purple conversion (Lanyi et al., 1992). Whether it was observed at low or high pH, only the purple form had a photocycle in which the Schiff base deprotonated, suggesting that both pK_a values refer to Glu-85. The two pK_a values were interpreted in terms of two coexisting conformations of the protein. In the Y185F mutant, illumination produces an increase in the amount of a quasi-stable blue species in which Asp-85 is protonated, even in the neutral pH range (Sonar et al., 1993). The explanation offered for this and the observed pH dependence of the effect (He et al., 1993; Rath et al., 1993) was that in Y185F the pK_a of Asp-85 is 3.7 when the retinal is 13-*cis* (in the dark-adapted state) but rises to approximately 9 when it is all-*trans* (in the light-adapted state).

The elevated pK_a of Asp-85 in Arg-82 mutants is now understood in the terms originally proposed. However, the results with D85E and Y185F needed to be reinvestigated in view of a recent model in which the pK_a of Asp-85 is controlled by the protonation state of another residue, Glu-204, in a way relevant to the mechanism of proton transport (Brown et al., 1995; Balashov et al., 1995a, 1996; Richter et al., 1996a). Because of this linkage, the titration of Asp-85

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Address reprint requests to Dr. Janos K. Lanyi, Department of Physiology and Biophysics, University of California, Irvine, CA 92697. Tel.: 714-824-7150; Fax: 714-824-8540; E-mail: jlanyi@orion.oac.uci.edu.

Terms used: mutants are referred to with the original residue name, followed by its number and the changed residue name, e.g., Y185F. M and O refer to two of the photointermediates of bacteriorhodopsin, bis-tris-propane and 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane.

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is biphasic in the wild type (but not in the E204Q mutant), although this is not immediately evident because the amplitude of the blue-to-purple shift associated with the higher of the two pK_a values is very small. Do the results with the D85E and Y185F mutants represent perturbation of this kind of interaction of Asp-85 and Glu-204, which changes quantitatively but not qualitatively the titration behavior of residue 85? Balashov et al. (1995a) suggested that the biphasic titration of D85E would be consistent with the Asp-85/Glu-204 model. We report results here that demonstrate that this is indeed so, and thereby give a simple and common explanation for the anomalous behavior of the Y185F and D85E mutants.

MATERIALS AND METHODS

Purple membranes were prepared from *Halobacterium salinarium* by a standard method. The Y185F, Y185F/R82Q, D85N/Y185F, Y185F/D96N, Y185F/E204Q, D85E, D85E/E204Q, and D85E/Y185F mutants have been constructed as described before (Needleman et al., 1991). Stationary spectra were measured in a Shimadzu model 1601 spectrophotometer connected to a desktop computer. Absorption changes at 405 nm were followed after photoexcitation with a Nd-Yag laser pulse (532 nm, 7 ns), as in earlier publications of ours (e.g., Váró et al., 1995). Light adaptation was for 5 min with white light. All samples were encased in polyacrylamide gels equilibrated with 100 mM NaCl, 20 mM phosphate, 10 mM bis-tris-propane buffer, at the pH specified. The temperature was 22°C throughout.

RESULTS

Spectroscopic titration of Glu-85 in the D85E mutant

Fig. 1 shows difference spectra of the chromophore, recorded after either progressively lowering (Fig. 1 *A*) or raising (Fig. 1 *B*) the pH from 7. In the former case, the red shift of the maximum that produces a blue state from the purple and, in the latter, the blue shift that depletes a blue state are evident. The somewhat different spectral shapes in the two pH regions (note the shift of the isosbestic point in Fig. 1 *B* toward a shorter wavelength) indicate that different species are interconverted in the upward and downward titrations. The pH dependence of these changes identifies two pK_a values, as reported before (Lanyi et al., 1992), but the amplitudes of the spectral changes are not strictly comparable in the upward and downward titrations and could not be used directly to construct a single titration curve for the group that deprotonates.

To connect the two parts of the titration, we used the rationale that the appearance of the M photointermediate is a direct measure of the availability of residue 85 as proton acceptor. Indeed, photoexcitation and measurement of amplitude of absorption change at 410 nm (not shown, but see Fig. 2B in Lanyi et al., 1992) indicated that qualitatively, at least, the more red-shifted state was present in the biphasic titration curve the less deprotonation of the Schiff base occurred in the photocycle. This is as expected, as the two spectral changes replace the purple-to-blue transition that occurs at much lower pH in the wild-type protein, and in

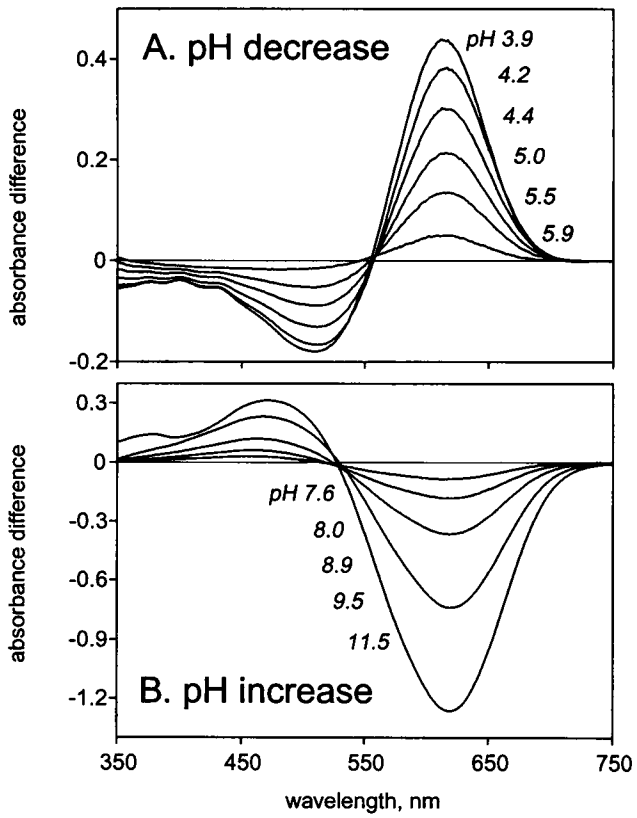
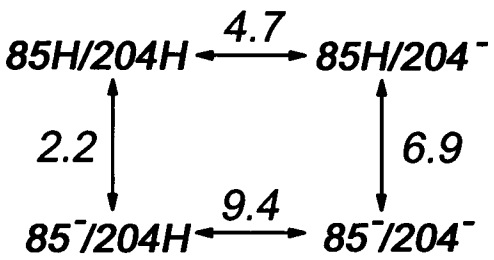


FIGURE 1 Titration of D85E bacteriorhodopsin from pH 7 downward (*A*) and upward (*B*). The difference spectra are between the pH of the measurement, as indicated, and pH 7. Bacteriorhodopsin, 36 μM.

that case, the protonation of Asp-85 has been shown to underlie the color shift (Subramaniam et al., 1990; Metz et al., 1992). As the M state is formed far more rapidly than it decays, its amplitude should be in fact a good quantitative indicator for the fraction of the protein with a functional proton acceptor, i.e., the unprotonated Glu-85 in this case. The relative amplitudes of the M state below and above neutral pH were used, therefore, to define the fraction of blue state in the saddle region of the titration curve near neutral pH and thus to scale the upward and downward absorption changes. The very small extent of Schiff base deprotonation above pH 8 that occurs independently from residue 85 through loss of the proton through the cytoplasmic proton channel (Kataoka et al., 1994) and the possibility of anomalous states at alkaline pH that do not produce an M intermediate were ignored. From such considerations, the titration data in Fig. 1 in the acid and alkaline directions were used to calculate a single titration curve explicitly for Glu-85 (Fig. 2 *A*). Little or no difference was observed in this curve when dark-adapted and light-adapted proteins were compared (not shown).

The titration curve in Fig. 2 *A* was fitted to the model (Balashov et al., 1995a, 1996) in which the pK_a of residue 85 depends on the protonation of another residue, recently

identified as Glu-204, and vice versa. The scheme therefore defines four pK_a values as follows:



Scheme 1

The two apparent pK_a values and the amplitude of the second component in the titrations define the three independent intrinsic pK_a values in this scheme. The line in Fig. 2 *A* is from a nonlinear least-squares fit of the model to the points. The flat, pH-independent saddle in the curve near neutral pH explains why the titration could be separated into a low and a high pH component. The calculated four pK_a values, for Glu-85 with protonated and unprotonated Glu-204 and for Glu-204 with protonated and unprotonated Glu-85, are given in Table 1. This model could explain the different shapes of the titration in the acid and alkaline directions; in the acid direction, the spectra refer to Glu-85H/Glu-204H minus the middle pH range mixture of Glu-85H/Glu-204$^-$ and Glu-85$^-$/Glu-204H near neutral pH, whereas in the alkaline direction, the spectra refer to Glu-85$^-$/Glu-204$^-$ minus the same mixture of Glu-85H/Glu-204$^-$ and Glu-85$^-$/Glu-204H. As expected, according to this model, the much more obvious biphasic titration than in the wild type originates mostly from the higher pK_a of Glu-85 in comparison with Asp-85 (4.8 vs. 2.2). If this is so, replacing Glu-204 with glutamine will eliminate the biphasic titration although not the elevated pK_a of Glu-85. Fig. 2 *B* shows the same titration as in Fig. 2 *A* but for the D85E/E204Q double mutant. Indeed, in this mutant, residue 85 titrates with a single pK_a. The difference between the calculated pK_a for Glu-85 when Glu-204 is protonated (i.e., 4.8) and when residue 204 is a glutamine (i.e., 6.7) suggests that some influence of residue 204 on residue 85, probably through hydrogen bonding via bound water (Scharnagl et al., 1995; Richter et al., 1996a), persists even when it is uncharged.

Spectroscopic titration of Asp-85 in the Y185F mutant

From an earlier report (Sonar et al., 1993), we expected that the spectroscopic titration of Y185F should be similar to that of D85E, but in addition, light adaptation causes a large increase in concentration of the state with protonated Asp-85. This interpretation relies on Fourier transform infrared spectroscopy (FTIR) difference spectra that had indicated the appearance of the 1762 cm^{-1} C=O stretch band of the

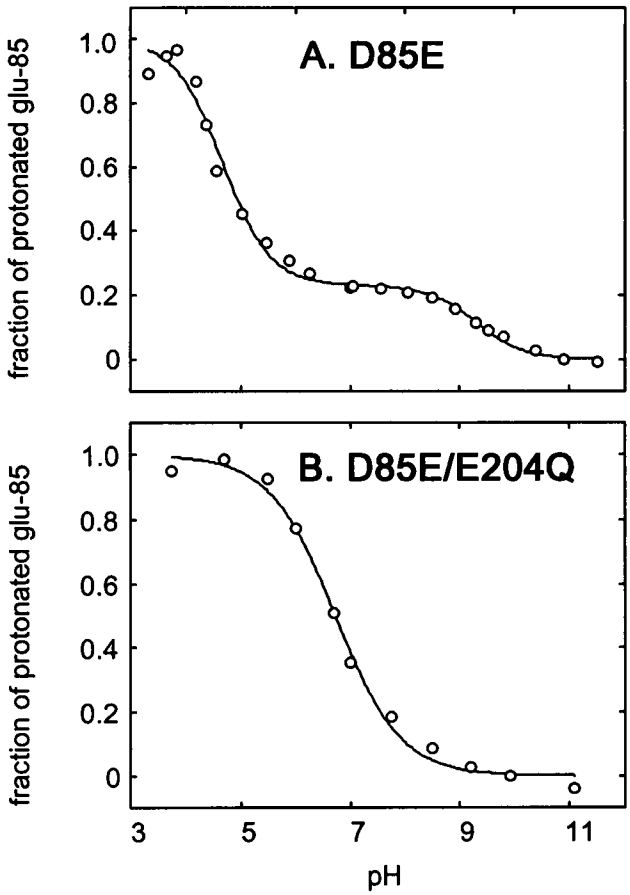


FIGURE 2 Titration curves for pH-dependent spectroscopic interconversions in D85E and D85E/E204Q bacteriorhodopsins (*A* and *B*, respectively). The lines are the best fits of the four-state model in Scheme 1.

protonated Asp-85 (Rath et al., 1993) upon the illumination, a finding that we confirmed (J. Sasaki, H. Kandori, A. Maeda, H-T. Richter, and J.K. Lanyi, unpublished results). It is not clear why other FTIR features otherwise typical of the O photointermediate of the photocycle, such as more intense hydrogen out-of-plane (hoop) bands, had also appeared upon light adaptation (cf. Discussion). Light-adapted blue membranes in the wild-type protein do not exhibit the hoop bands (Smith and Mathies, 1985). Fig. 3, *A* and *B*, show difference spectra in titrations toward lower and higher pH in the dark-adapted state (as in Fig. 1, *A* and *B*), and Fig. 4, *A* and *B*, shows analogous spectra in the light-adapted state. The shapes of the two sets of difference spectra are similar in the two cases, except for some increase in absorption in the blue region in the illuminated samples. The amplitudes in this mutant are affected not only by pH, however, but also by the isomeric state of the retinal. In the light-adapted state a correlation between the amplitude of M state (with unprotonated Asp-85) and the spectral amplitudes in the low and high pH regions could be obtained, as for D85E, and utilizing this relationship also for the data from dark-adapted samples produced the titration curves for Asp-85 in Fig. 5 *A*. The essential difference

TABLE 1 Calculated pK_a values for residues 85 and 204 in wild-type bacteriorhodopsin and various mutants

	pK_a of residue 85		pK_a of residue 204	
	Residue 204 protonated	Residue 204 anionic	Residue 85 protonated	Residue 85 anionic
Wild type	2.2	6.9	4.7	9.4
D85E	4.8	8.7	5.3	9.2
D85E/E204Q	6.7			
Y185F (dark adapted)	3.5	9.5	4.4	10.4
Y185F (light adapted)	4.3	9.6	4.5	9.7
Y185F/E204Q	5.5			

The values for wild type are from Richter et al. (1996), which are essentially as in Balashov et al. (1996). The other numbers represent nonlinear least-square best fits to the titrations in Figs. 2 and 5, as shown. Somewhat better fits were obtained when the number of protons was allowed to be different from 1 (not shown).

caused by light adaptation is that more of the species with protonated Asp-85 is present than in the dark-adapted protein as reported before (Rath et al., 1993), but we now find that it is a consequence of a modification of the biphasic titration.

These titrations could be fitted to the Asp-85/Glu-204-linked protonation state model, as indicated by the lines in Fig. 5 *A*. The calculated four pK_a values in Table 1 indicate that in Y185F, as in D85E, the fact that the pK_a of residue 85 is higher is the cause of the more obvious anomalous titration than in the wild type. Light adaptation increases this pK_a further by approximately 1 pH unit. The species, termed the O-like state (Duñach et al., 1990; He et al., 1993; Rath et al., 1993; Sonar et al., 1993), is identified by this model as Asp-85H/Glu-204$^-$. This indeed describes the O intermediate in the photocycle insofar as the protonation states of Asp-85 and the isomeric state of the retinal (all-*trans*) with a protonated Schiff base are concerned. Perhaps the O-like additional vibrational modes of the retinal are consequences of these properties. We report elsewhere that the distortion of the retinal chain, which causes the high amplitude hydrogen out-of-plane vibrational bands of the O state, does not relax until Asp-85 deprotonates at the end of the photocycle (Richter et al., 1996b).

The model is again critically tested by the phenotype of the Y185F/E204Q double mutant. As shown in Fig. 5 *B*, it exhibits a single pK_a, consistent with the proposed role of Glu-204 in the biphasic titration. A similar result was obtained for Y185F/R82Q (not shown), where the interaction of Asp-85 and Glu-204 is disrupted by replacement of Arg-82 (Balashov et al., 1995a). On the other hand, D85N/Y185F bacteriorhodopsin had a blue chromophore (maximum at 610 nm, as in D85N) that did not change when the pH was raised up to 10, apart from a shift to 410 nm that occurs when the Schiff base deprotonates as in D85N (cf. below). This is consistent with the idea that the titration curves in Fig. 5 *A* refer to protonation of Asp-85. A negative control was provided in turn by Y185F/D96N. The similarity of its light-adaptation behavior to Y185F (not shown) indicated that, when Asp-85 becomes protonated in the O-like state, Asp-96 is not the proton donor.

Spectroscopic titration of Glu-85 in the D85E/Y185F double mutant

If the D85E and Y185F mutations both increase the pK_a of residue 85, they must do so through different mechanisms. We expected therefore that the postulated pK_a increases caused by each mutation will be additive. Indeed, according to the spectroscopic titration of the D85E/Y185F double mutant, the blue color of the chromophore (maximum at 600 nm) persisted up to at least pH 10 (Fig. 6). In the pH 8–10 region, instead of transition to a purple chromophore, which would indicate deprotonation of Glu-85, there was a gradual shift to near 500 nm as at higher pH in the wild type (Balashov et al., 1991). Above pH 11, the retinal chromophore was lost. The kinetics of M after photoexcitation is another indicator of the anionic state of residue 85. Up to pH 9.5, only a small amount of M photointermediate was observed, and with slow (3–4 ms) rise kinetics similar to that in the D85N mutant where the proton is released to the cytoplasmic side (Tittor et al., 1994; Kataoka et al., 1994). Above this pH, the amplitude of M increased somewhat and its rise became as fast (a few microseconds) as in the D85E single mutant (Lanyi et al., 1992), indicating that only at this pH and above does residue 85 begin to function as a proton acceptor. There is no evidence that the Schiff base becomes deprotonated before the chromophore is lost.

Spectroscopic titration of the Schiff base in the D85N/Y185F double mutant

In D85N, the Schiff base pK_a is lowered from above 13 (Druckmann et al., 1982) to 9.2 (Otto et al., 1990; Brown et al., 1993; Tittor et al., 1994; Kataoka et al., 1994). The perturbation of Asp-85 in the Y185F mutant might be expected to perturb this pK_a as well. Fig. 7 compares titration curves of D85N and D85N/Y185F based on the shift of the absorption maximum of the chromophore from near 600 nm to 405 nm when the Schiff base is deprotonated. The pK_a of the Schiff base is raised from 9.3 to 10.6 in the double mutant. Furthermore, whereas the titration of D85N is with an apparent proton stoichiometry of 0.5, probably caused by the influence of Arg-82, which also

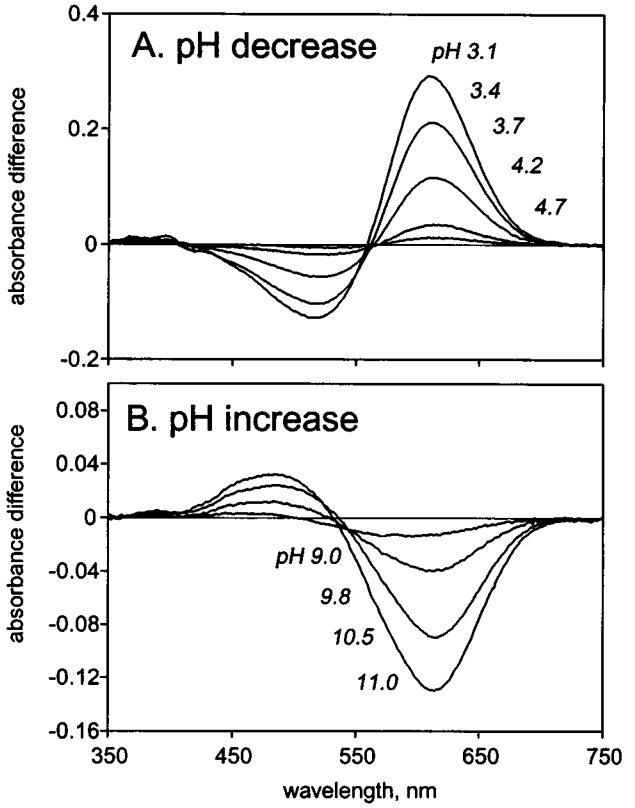


FIGURE 3 Titration of dark-adapted Y185F bacteriorhodopsin from pH 7 downward (*A*) and upward (*B*). The difference spectra are between the pH of the measurement, as indicated, and pH 7. Bacteriorhodopsin, 26 μM.

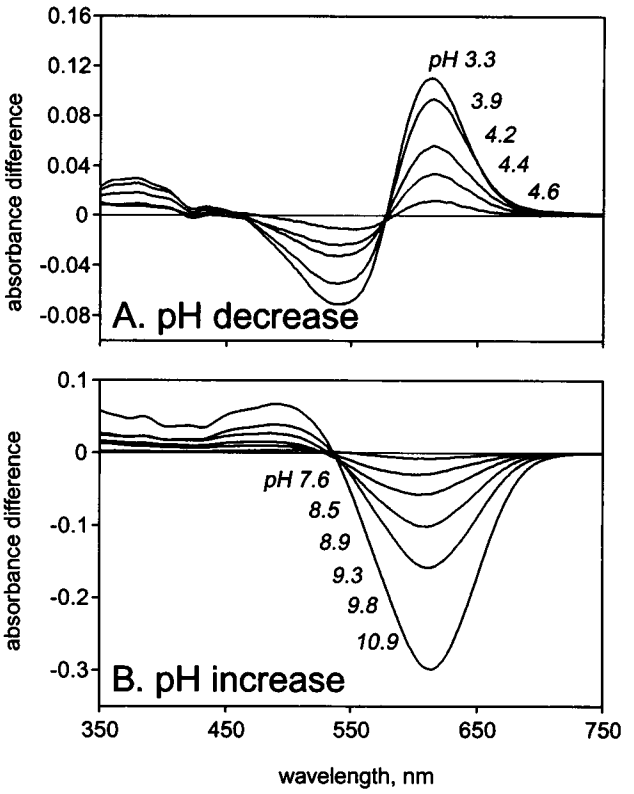


FIGURE 4 Titration of light-adapted Y185F bacteriorhodopsin from pH 7 downward (*A*) and upward (*B*). The difference spectra are between the pH of the measurement, as indicated, and pH 7. Bacteriorhodopsin, 26 μM.

deprotonates in this pH range (Brown et al., 1993), in D85N/Y185F the stoichiometry is 1.

DISCUSSION

We have demonstrated here that the anomalous titration behavior reported earlier in the D85E and Y185F mutants (Lanyi et al., 1992; He et al., 1993; Rath et al., 1993; Sonar et al., 1993) originates from the raised pK_a for residue 85 and the perturbation of the influence of Glu-204 on this pK_a. The biphasic character of the titration curve itself is not different from the wild type. What is different is the greatly increased amount of the species with protonated residue 85 in the pH region between the first and the second pK_a and the increase of the amount of this species in Y185F upon light adaptation. The titration curves fit the earlier proposed (Balashov et al., 1995a, 1996; Richter et al., 1996a) scheme for the mutual effect of residue 85 and Glu-204 on one another (Figs. 2 *A* and 5). As expected, the higher pK_a values disappear in the D85E/E204Q and Y185F/E204Q (as well as the Y185F/R82Q and Y185F/D85N) double mutants (Figs. 2 *B* and 5 *B*), in which the influence of Glu-204 on residue 85 is necessarily absent.

Table 1 shows the calculated pK_a values of Asp-85 and Glu-204 in the wild type and various mutants. Strikingly, it is the pK_a of residue 85 that is most variable and accounts for the more obvious biphasic titration curves. The increased pK_a in the light-adapted Y185F sample, which contains all-*trans* rather than 13-*cis*,15-*syn* retinal, requires special mention. The acute angle of the polyene chain in C_{13}-C_{14} *cis* retinal is avoided with the additional rotation of the C_{15}-N double bond, and the overall shape of the retinal is not very different from that of the linear all-*trans* (Smith et al., 1984). Evidently, even though it is small, the displacement of the Schiff base upon isomerization from nearly 100% 13-*cis*,15-*syn* (Rath et al., 1993) to all-*trans* during light adaptation is sufficient to raise the pK_a of Asp-85 by approximately 1 unit. This is reasonable considering the close proximity of the Schiff base and Asp-85. A smaller but distinct increase of the pK_a of Asp-85 was noted upon light adaptation of wild type bacteriorhodopsin (Balashov et al. 1995b).

The nature of the perturbation of the Asp-85/Glu-204 region by the two mutations studied remains unclear until the structure is known to a higher resolution. Speculations as to the cause of the perturbed interaction must be based on the interaction of residue 85 and the protonated Schiff base as well as the suggested role of hydrogen-bonded water that connects residues 85 and 204. It seems likely that replacing Asp-85 with a glutamate moves the carboxylate into a different environment where its geometry to the Schiff base and bound water will have changed. Removing a phenolic

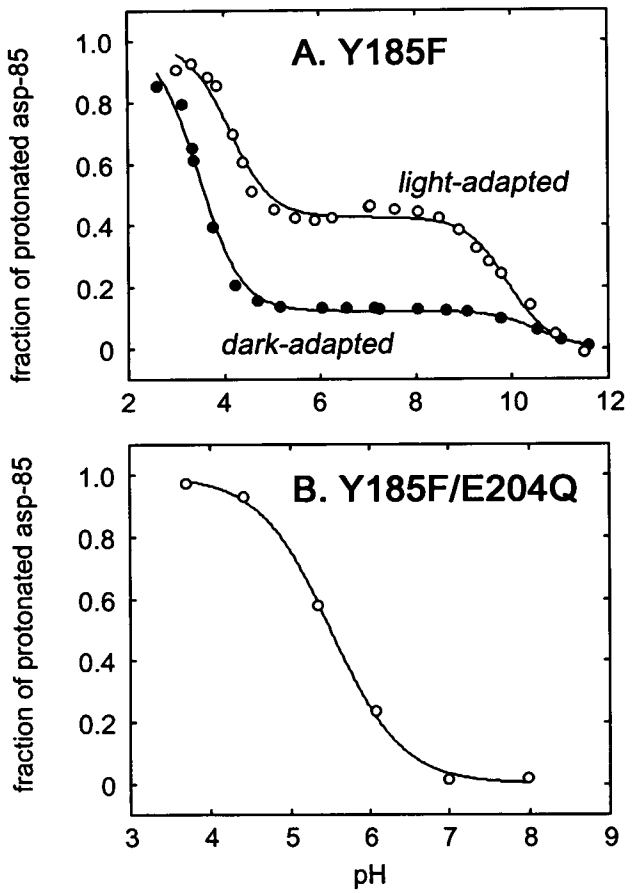


FIGURE 5 Titration curves for pH-dependent spectroscopic interconversions in dark-adapted (●) and light-adapted (○) Y185F (*A*) and Y185F/E204Q bacteriorhodopsins (*B*). The lines are the best fits of the four-state model in Scheme 1.

hydroxyl upon replacing Tyr-185 with phenylalanine would affect Asp-212 and its interaction with the Schiff base (cf. below) and might also change the water network that leads

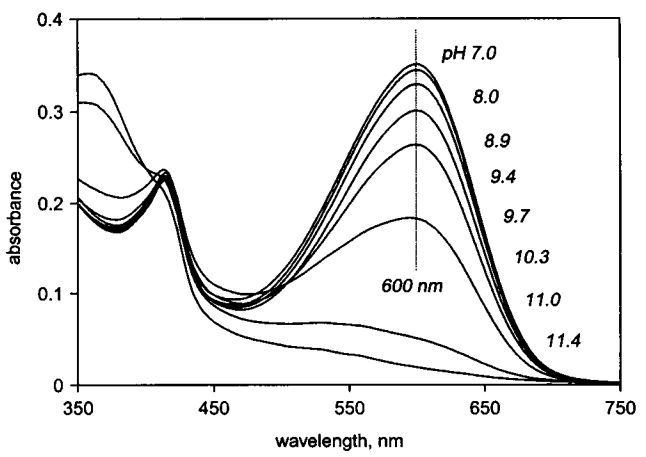


FIGURE 6 Absorption spectra for D85E/Y185F bacteriorhodopsin as a function of pH, as indicated.

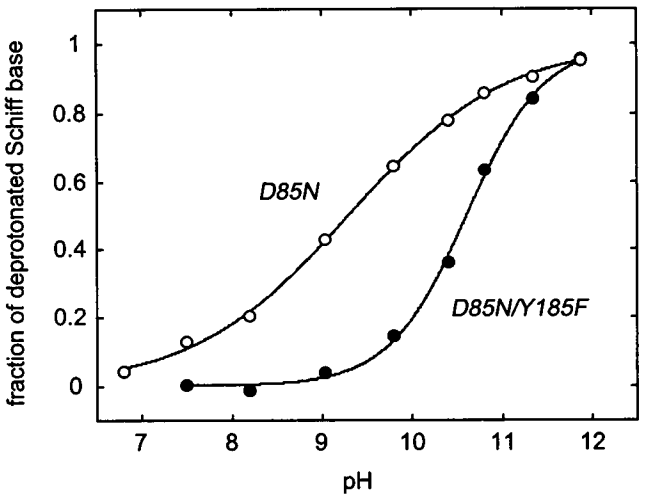


FIGURE 7 Titration curves for the deprotonation of the retinal Schiff base in D85N (○) and D85N/Y185F (●) bacteriorhodopsins. The fractional amounts of deprotonated Schiff base are from the amplitudes of the pH-dependent difference spectra (not shown). Lines are nonlinear best fits with pK_a = 9.3 (n = 0.51) for D85N and pK_a = 10.6 (n = 1.0) for D85N/Y185F.

to Glu-204. In any case, both mutations change the pK_a of residue 85 more than the pK_a of residue 204 (Table 1).

The unusually slow rate of dark adaptation in Y185F (Sonar et al., 1993), which contains protonated Asp-85, appears to contradict the relationship established between the protonation state of Asp-85 and the thermal equilibration of the retinal between all-*trans* and 13-*cis*,15-*anti*; when Asp-85 is protonated, dark adaptation should be rapid (Balashov et al., 1993). The observed rise in the pK_a of the Schiff base when Tyr-185 is changed to phenylalanine (Fig. 7) provides a clue as to the reason for this. It may be the increased counterion strength of the anionic Asp-212, when the hydrogen-bonding of the latter with the phenolic hydroxyl of Tyr-185 (Duñach et al., 1990; Rath et al., 1993) is eliminated. Stronger interaction with the anionic Asp-212 should not only raise the Schiff base pK_a as observed (Fig. 7) but also counteract the postulated lowering of the barrier to thermal isomerization when Asp-85 is uncharged. In fact, this stronger interaction of the Schiff base with Asp-212 in the Y185F mutant may be what increases the pK_a of Asp-85 to a value more typical for an aspartate. This possibility is supported by the observation (L.S. Brown, H-T. Richter, R. Needleman, and J.K. Lanyi, unpublished results) that the characteristic Y185F phenotype is not evident in the D212N/Y185F mutant.

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REFERENCES

Balashov, S. P., R. Govindjee and T. G. Ebrey. 1991. Red shift of the purple membrane absorption band and the deprotonation of tyrosine residues at high pH. *Biophys. J.* 60:465–490.

Balashov, S. P., R. Govindjee, E. S. Imasheva, S. Misra, T. G. Ebrey, Y. Feng, R. K. Crouch, and D. R. Menick. 1995a. The two pK_a's of aspartate-85 and control of thermal isomerization and proton release in the arginine-82 to lysine mutant of bacteriorhodopsin. *Biochemistry.* 34:8820–8834.

Balashov, S. P., R. Govindjee, E. S. Imasheva, M. Sheves, and T. G. Ebrey. 1995b. pK_a of Asp85 is higher in all-*trans* than in 13-*cis* bacteriorhodopsin. *Biophys. J* 68:A334.

Balashov, S. P., R. Govindjee, M. Kono, E. Imasheva, E. Lukashev, T. G. Ebrey, R. K. Crouch, D. R. Menick, and Y. Feng. 1993. Effect of the arginine-82 to alanine mutation in bacteriorhodopsin on dark adaptation, proton release, and the photochemical cycle. *Biochemistry.* 32: 10331–10343.

Balashov, S. P., E. S. Imasheva, R. Govindjee, and T. G. Ebrey. 1996. Titration of aspartate-85 in bacteriorhodopsin: what it says about chromophore isomerization and proton release. *Biophys. J.* 70:473–481.

Braiman, M. S., T. Mogi, T. Marti, L. J. Stern, H. G. Khorana, and K. J. Rothschild. 1988. Vibrational spectroscopy of bacteriorhodopsin mutants: light-driven proton transport involves protonation changes of aspartate residues 85, 96, and 212. *Biochemistry.* 27:8516–8520.

Brown, L. S., L. Bonet, R. Needleman, and J. K. Lanyi. 1993. Estimated acid dissociation constants of the Schiff base, Asp-85, and Arg-82 during the bacteriorhodopsin photocycle. *Biophys. J.* 65:124–130.

Brown, L. S., J. Sasaki, H. Kandori, A. Maeda, R. Needleman, and J. K. Lanyi. 1995. Glutamic acid 204 is the terminal proton release group at the extracellular surface of bacteriorhodopsin. *J. Biol. Chem.* 270: 27122–27126.

Druckmann, S., M. Ottolenghi, A. Pande, J. Pande, and R. H. Callender. 1982. Acid-base equilibrium of the Schiff base in bacteriorhodopsin. *Biochemistry.* 21:4953–4959.

Duñach, M., S. Berkowitz, T. Marti, Y-W. He, S. Subramaniam, H. G. Khorana, and K. Rothschild. 1990. Ultraviolet-visible transient spectroscopy of bacteriorhodopsin mutants. *J. Biol. Chem.* 265:16978–16984.

Fischer, U., and D. Oesterhelt. 1979. Chromophore equilibria in bacteriorhodopsin. *Biophys. J.* 28:211–230.

Greenhalgh, D. A., S. Subramaniam, U. Alexiev, H. Otto, M. P. Heyn, and H. G. Khorana. 1992. Effect of introducing different carboxylate-containing side chains at position 85 on chromophore formation and proton transport in bacteriorhodopsin. *J. Biol. Chem.* 267:25734–25738.

He, Y., M. P. Krebs, W. B. Fischer, H. G. Khorana, and K. J. Rothschild. 1993. FTIR difference spectroscopy of the bacteriorhodopsin mutant Tyr-185→Phe: detection of a stable O-like species and characterization of its photocycle at low temperature. *Biochemistry.* 32:2282–2290.

Jonas, R., and T. G. Ebrey. 1991. Binding of a single divalent cation directly correlates with the blue-to-purple transition in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 88:149–153.

Kataoka, M., H. Kamikubo, F. Tokunaga, L. S. Brown, Y. Yamazaki, A. Maeda, M. Sheves, R. Needleman, and J. K. Lanyi. 1994. Energy coupling in an ion pump: the reprotonation switch of bacteriorhodopsin. *J. Mol. Biol.* 243:621–638.

Lanyi, J. K., J. Tittor, G. Váró, G. Krippahl, and D. Oesterhelt. 1992. Influence of the size and protonation state of acidic residue 85 on the absorption spectrum and photoreaction of the bacteriorhodopsin chromophore. *Biochim. Biophys. Acta.* 1099:102–110.

Marti, T., S. J. Rösselet, H. Otto, M. P. Heyn, and H. G. Khorana. 1991. The retinylidene Schiff base counterion in bacteriorhodopsin. *J. Biol. Chem.* 266:18674–18683.

Metz, G., F. Siebert, and M. Engelhard. 1992. Asp^{85} is the only internal aspartic acid that gets protonated in the M intermediate and the purple-to-blue transition of bacteriorhodopsin: a solid-state ^{13}C CP-MAS NMR investigation. *FEBS Lett.* 303:237–241.

Miercke, L. J. W., M. C. Betlach, A. K. Mitra, R. F. Shand, S. K. Fong, and R. M. Stroud. 1991. Wild-type and mutant bacteriorhodopsins D85N, D96N, and R82Q: purification to homogeneity, pH dependence of pumping, and electron diffraction. *Biochemistry.* 30:3088–3098.

Mowery, P. C., R. H. Lozier, Q. Chae, Y. W. Tseng, M. Taylor, and W. Stoeckenius. 1979. Effect of acid pH on the absorption spectra and photoreactions of bacteriorhodopsin. *Biochemistry.* 18:4100–4107.

Needleman, R., M. Chang, B. Ni, G. Váró, J. Fornes, S. H. White, and J. K. Lanyi. 1991. Properties of asp212-asn bacteriorhodopsin suggest that asp212 and asp85 both participate in a counterion and proton acceptor complex near the Schiff base. *J. Biol. Chem.* 266:11478–11484.

Otto, H., T. Marti, M. Holz, T. Mogi, L. J. Stern, F. Engel, H. G. Khorana, and M. P. Heyn. 1990. 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. *Proc. Natl. Acad. Sci. USA.* 87:1018–1022.

Rath, P., M. P. Krebs, Y. He, H. G. Khorana, and K. J. Rothschild. 1993. Fourier transform Raman spectroscopy of the bacteriorhodopsin mutant Tyr-185→Phe: formation of a stable O-like species during light adaptation and detection of its transient N-like photoproduct. *Biochemistry.* 32:2272–2281.

Richter, H. T., L. S. Brown, R. Needleman, and J. K. Lanyi. 1996a. A linkage of the pK_a's of Asp-85 and Glu-204 forms part of the reprotonation switch of bacteriorhodopsin. *Biochemistry.* 35:4054–4062.

Richter, H. T., R. Needleman H. Kandori, A. Maeda, and J. K. Lanyi. 1996b. Relationship of retinal configuration and internal proton transfer at the end of the bacteriorhodopsin photocycle. *Biochemistry.* In press.

Scharnagl, C., J. Hettenkofer, and S. F. Fischer. 1995. Electrostatic and conformational effects on the proton translocation steps in bacteriorhodopsin: analysis of multiple M structures. *J. Phys. Chem.* 99:7787–7800.

Smith, S. O., and R. A. Mathies. 1985. Resonance Raman spectra of the acidified and deionized forms of bacteriorhodopsin. *Biophys. J.* 47: 251–254.

Smith, S. O., A. B. Myers, J. A. Pardoen, C. Winkel, P. P. J. Mulder, J. Lugtenburg, and R. A. Mathies. 1984. Determination of retinal Schiff base configuration in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 81:2055–2059.

Sonar, S., M. P. Krebs, H. G. Khorana, and K. J. Rothschild. 1993. Static and time-resolved absorption spectroscopy of the bacteriorhodopsin mutant Tyr-185→Phe: evidence for an equilibrium between bR_{570} and an O-like species. *Biochemistry.* 32:2263–2271.

Stern, L. J., and H. G. Khorana. 1989. Structure-function studies on bacteriorhodopsin. X. Individual substitutions of arginine residues by glutamine affect chromophore formation, photocycle, and proton translocation. *J. Biol. Chem.* 264:14202–14208.

Subramaniam, S., D. A. Greenhalgh, and H. G. Khorana. 1992. Aspartic acid 85 in bacteriorhodopsin functions both as proton acceptor and negative counterion to the Schiff base. *J. Biol. Chem.* 267:25730–25733.

Subramaniam, S., T. Marti, and H. G. Khorana. 1990. Protonation state of Asp (Glu)-85 regulates the purple-to-blue transition in bacteriorhodopsin mutants Arg-82→Ala and Asp-85→Glu: the blue form is inactive in proton translocation. *Proc. Natl. Acad. Sci. USA.* 87:1013–1017.

Tittor, J., U. Schweiger, D. Oesterhelt, and E. Bamberg. 1994. Inversion of proton translocation in bacteriorhodopsin mutants D85N, D85T, and D85,D96N. *Biophys. J.* 67:1682–1690.

Váró, G., and J. K. Lanyi. 1989. Photoreactions of bacteriorhodopsin at acid pH. *Biophys. J.* 56:1143–1151.

Váró, G., R. Needleman, and J. K. Lanyi. 1996. Protein structural change at the cytoplasmic surface as the cause of cooperativity in the bacteriorhodopsin photocycle. *Biophys. J.* 70:461–467.