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Titration of Aspartate-85 in Bacteriorhodopsin: What It Says About Chromophore Isomerization and Proton Release

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ABSTRACT Titration of Asp-85, the proton acceptor and part of the counterion in bacteriorhodopsin, over a wide pH range (2-11) leads us to the following conclusions: 1) Asp-85 has a complex titration curve with two values of pK_a; in addition to a main transition with pK_a = 2.6 it shows a second inflection point at high pH (pK_a = 9.7 in 150-mM KCl). This complex titration behavior of Asp-85 is explained by interaction of Asp-85 with an ionizable residue X'. As follows from the fit of the titration curve of Asp-85, deprotonation of X' increases the proton affinity of Asp-85 by shifting its pK_a from 2.6 to 7.5. Conversely, protonation of Asp-85 decreases the pK_a of X' by 4.9 units, from 9.7 to 4.8. The interaction between Asp-85 and X' has important implications for the mechanism of proton transfer. In the photocycle after the formation of M intermediate (and protonation of Asp-85) the group X' should release a proton. This deprotonated state of X' would stabilize the protonated state of Asp-85. 2) Thermal isomerization of the chromophore (dark adaptation) occurs on transient protonation of Asp-85 and formation of the blue membrane. The latter conclusion is based on the observation that the rate constant of dark adaptation is directly proportional to the fraction of blue membrane (in which Asp-85 is protonated) between pH 2 and 11. The rate constant of isomerization is at least 10^4 times faster in the blue membrane than in the purple membrane. The protonated state of Asp-85 probably is important for the catalysis not only of all-*trans* ⇔ 13-*cis* thermal isomerization during dark adaptation but also of the reisomerization of the chromophore from 13-*cis* to all-*trans* configuration during N → O → bR transition in the photocycle. This would explain why Asp-85 stays protonated in the N and O intermediates.

INTRODUCTION

The cyclic process of the light energy transduction by bacteriorhodopsin (bR) of the purple membrane from *Halobacterium salinarium* (Oesterhelt and Stoeckenius, 1971) includes several consecutive steps: all-*trans* → 13-*cis* photoisomerization of the chromophore of bR, proton transfer from the chromophore (the protonated Schiff base) to the internal proton acceptor Asp-85, and subsequent proton release to the extracellular side of the purple membrane. This is followed by reprotonation of the Schiff base from the cytoplasmic side, 13-*cis* → *trans* thermal reisomerization of the chromophore and reformation of the initial pigment. All the stages of the process are controlled and catalyzed by the protein (see, for reviews, Oesterhelt et al., 1992; Rothschild, 1992; El-Sayed, 1993; Ebrey, 1993; Lanyi, 1993; Balashov and Ebrey, 1994). Many aspects of this control remain obscure. In our previous studies of dark adaptation in R82A and R82K mutants of bR, we found evidence for a strong link between the rate of thermal isomerization of the chromophore and the protonation state of Asp-85 (Balashov et al., 1993, 1995). These results and earlier suggestions of Warshel and Ottolenghi (1979) indicate that the pH dependence of thermal isomerization (dark adaptation) in bR may be an important source of information on the chromophore-protein interaction, on the mechanism controlling the proton affinity of Asp-85 (pK_a of Asp-85), and on the interaction of Asp-85 with other residues presumably involved in the proton transfer.

Asp-85 is deprotonated in the purple membrane, but when the pH is decreased it becomes protonated (Subramaniam et al., 1990; Metz et al., 1992) (pK_a = 2.6 in 150-mM KCl), which causes formation of the red-shifted species called the blue membrane (Fischer and Oesterhelt, 1979; Mowery et al., 1979; Szundi and Stoeckenius, 1987; Jonas et al., 1990), which is incapable of light-induced proton transfer. In the photocycle of functionally active purple membrane the pK_a of Asp-85 transiently increases; it undergoes protonation during formation of the M intermediate and stays protonated in N and O intermediates, finally deprotonating during reformation of bR (Braiman et al., 1988; Souvignier and Gerwert, 1992). Protonation of Asp-85 is followed by deprotonation of an unidentified group X (Zimányi et al., 1992; Ebrey, 1993; Lanyi, 1993), which releases a proton from the outer membrane surface. We have presented evidence that Arg-82 is X or at least part of a cluster of residues that constitute X (Balashov et al., 1993, 1995). Other residues that are presumably involved in the proton release process are Tyr-57 (Govindjee et al., 1995) and perhaps Glu-204 (Scharnagl et al., 1995). Asp-85 deprotonates during the O-to-bR conversion. Control of the pK_a of Asp-85 is an essential part of proton transfer; however, little is known about the mechanism of this process. It has been shown that the pK_a of Asp-85 is sensitive to the mutation of several amino acid residues. In particular, re-

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Abbreviations used: bR, bacteriorhodopsin; WT, wild type.

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placing positively charged Arg-82 with neutral alanine or glutamine has a strong effect on the pK_a of Asp-85, which increases by 4.6 pH units (Stern and Khorana, 1989; Subramaniam et al., 1990; Balashov et al., 1993; Brown et al., 1993).

The pH dependence of the rate constant of dark adaptation, k_{da} (thermal isomerization of the chromophore from the all-*trans*, 15-*anti* into the 13-*cis*, 15-*syn* configuration (Oesterhelt et al., 1973; Harbison et al., 1984)), in wild type (WT) bR has two transitions (Ohno et al., 1977) with pK_a = 2.9 and pK_a = 9.6 in 150-mM KCl (Balashov et al., 1993). This is drastically changed in the R82A mutant in which k_{da} has only one pK_a, 7.5, the same as the pK_a of Asp-85 in this mutant (Balashov et al., 1993). Moreover, the rate constant of thermal isomerization in R82A and R82K mutants is proportional to the fraction of protonated Asp-85, which suggests that thermal isomerization of the chromophore in R82 mutants occurs on protonation of Asp-85.

We have proposed that thermal isomerization of the chromophore proceeds through a transient protonation of Asp-85 not only in the R82A mutant but also in the WT (Balashov et al., 1993). To explain the complex pH dependence of the rate constant of thermal isomerization in the WT we suggested that Asp-85 also has a similar complex titration curve as the result of its interaction with an ionizable residue X′. This hypothesis predicts that the titration curve of Asp-85 should have two values of pK_a corresponding to the protonated and deprotonated states of X′ and that the rate constant of dark adaptation should be directly proportional to the fraction of protonated Asp-85.

We investigated the role of Asp-85 in the catalysis of thermal isomerization of the chromophore in the WT bR and tested the above hypothesis. We measured the fraction of blue membrane as a function of pH (between pH 2 and 11) and present evidence suggesting that the protonation state of Asp-85 is controlled by a second residue (X′) and that isomerization proceeds through the protonation of Asp-85.

MATERIALS AND METHODS

Purple membrane was isolated from the *Halobacterium salinarium* (former *halobium*) strain S9 as described previously. Protonation of Asp-85 was monitored by the formation of blue membrane, which was measured spectroscopically. The optical properties of the blue membrane at low pH have been characterized previously in several studies (Moore et al., 1978; Fischer and Oesterhelt, 1979; Mowery et al., 1979; Varo and Lanyi, 1989; Jonas and Ebrey, 1991). The unusual feature of this study is that the blue ⇔ purple transition was detected at high pH, where its fraction is very small. Moreover, at high pH other pH-dependent spectral transitions take place whose contributions have to be sorted out. This has been done by using optically dense samples and by monitoring the blue membrane at specific wavelengths (near 690 nm) at which the absorbance of purple membrane and the other spectral species is negligibly small compared with that of the blue membrane. This, together with recording the pH-induced difference spectra, enabled us to follow the pH dependence of the blue membrane up to pH = 11. The measurements of absorption spectra and kinetics of dark adaptation were done at 20°C with a Cary-Aviv spectrophotometer. The noise in the digital recording of the difference spectra was less than 0.1 mOD. Dark-adapted membranes were used. All the values of pK_a cited below are for 150-mM KCl. To maintain the pH between 2 and 11 during titration, a mixture of several buffers (citric acid, Mes, Mops, Tricine, Ches, and Caps), each at 5 mM, was used. The pH was changed by addition of 1-N KOH or H_2SO_4. Kinetics of dark adaptation was measured in both suspensions and polyacrylamide gels containing purple membranes (to prevent aggregation and sedimentation of membranes). Gels were prepared as described by Liu et al. (1991) and were incubated at a given pH for at least 12 h.

RESULTS

pH dependence of the rate constant of thermal isomerization (dark adaptation), k_{da}, in bR

We have extended the previous studies of the pH dependence of the rate constant of dark adaptation (Ohno et al., 1977; Balashov et al., 1993; Drachev et al., 1993) by trying to make accurate estimates of the values of pK_a of k_{da} and the maximal value of the rate constant at low pH, to compare the pH dependence of k_{da} with the fraction of blue membrane.

Fig. 1 *A* shows a set of difference spectra accompanying dark adaptation at pH 9. Similar absorption changes are

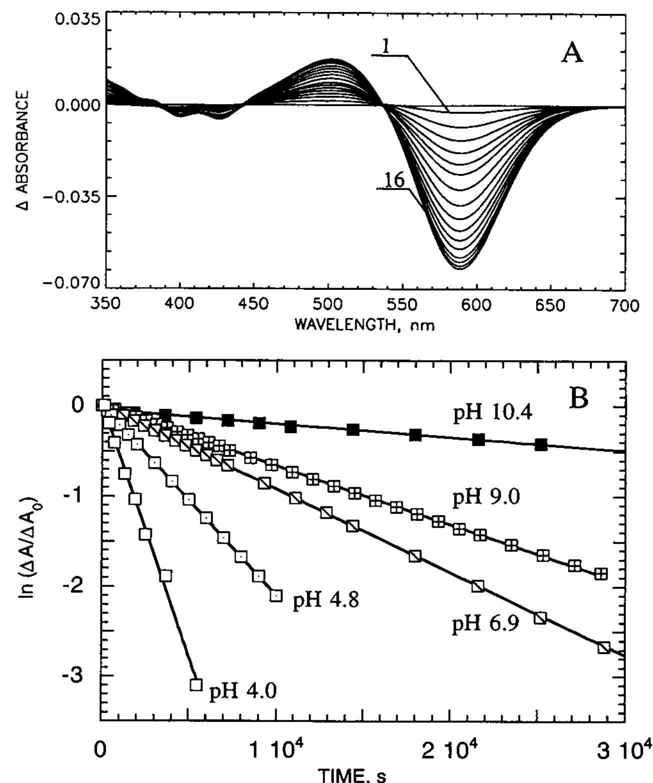

FIGURE 1 Absorption changes and kinetics of dark adaptation in a suspension of purple membrane (in 150-mM KCl, 20°C). (*A*) 1–16, difference absorption spectra taken 10, 30, 50, 70, 90, 110, 140, 180, 220, 260, 300, 340, 390, 420, 450, and 480 min after light adaptation at pH 9. (*B*) Kinetics of dark adaptation at different pH. Logarithm of absorption changes at 580 nm are plotted versus time. The rate constants k_{da} were determined from the fit of the data with the following equation: $\ln(\Delta A(t)/\Delta A_0) = -k_{da}t$, where $\Delta A(t) = A_{580}(t = 0) - A_{580}(t)$; ΔA_0 is the maximal absorbance change at 580 nm for the complete transition from the light-adapted to the dark-adapted state, as determined from the fit.

observed between pH 4.0 and pH 10.4. Kinetic analysis of the absorbance decay at 580 nm (after light adaptation) shows that dark adaptation proceeds as a first-order kinetic process between pH 4.0 and 10.4 (Fig. 1 *B*).

Below pH 3.5 the kinetics of the absorption changes at 580 nm in polyacrylamide gels containing purple membrane do not follow simple first-order kinetics and can be decomposed into two components (Fig. 2 *A*). One component may originate from dark adaptation of purple membrane (conversion of all-*trans*-purple into 13-*cis*-purple), whereas the other probably is associated with the photochemical reactions of the blue membrane at pH < 3.5. To exclude any contribution from the light-induced transformations of the blue membrane, we performed measurements in suspensions of purple membrane that were light adapted at pH 7, and then the pH of the suspension was decreased in the dark and the absorbance changes at 580 nm were measured. In this way we avoided illumination at low pH and did not induce photoreactions of blue membrane. Because the purple-to-blue transition has an isosbestic point close to 580 nm, the absorbance changes that were due to this transition did not contribute to the kinetics of absorption changes measured at 580 nm, which were due mainly to the transformation of all-*trans*-bR-purple to 13-*cis*-bR-purple. The kinetics of relaxation of the absorbance changes at 580 nm in suspensions that were light adapted at pH 7 and then adjusted to low pH were monoexponential. The rate constants of dark adaptation in suspensions (filled squares in Fig. 2 *A*) are close to the slow kinetic component observed in the gels that were light adapted at low pH (crossed squares). We conclude therefore that the slow component of the 580-nm absorbance change is due to the transformation of all-*trans*-purple to 13-*cis*-purple. The amplitude of the slow kinetic component decreases as the pH decreases, with a pK_a close to the pK_a of the purple-to-blue transition, which reflects the decrease in the fraction of the purple membrane (Fig. 2 *B*). In contrast to this, the fast component shows significant amplitude even at pH = 1.8 (where the fraction of purple membrane is less than 10%). Most likely it originates from some process other than the transformation of all-*trans*-bR-purple to 13-*cis*-bR-purple.

The pH dependence of the rate constant of thermal all-*trans* ⇔ 13-*cis* isomerization, k_{da}, between pH = 1.8 and pH = 10.4 is shown in Fig. 3 (squares). The maximum rate of 0.01 s^{-1} is observed at pH 1.8 (in 150-mM KCl at 20°C), where ~90% of pigment is in the form of blue membrane. It corresponds to a life time of 1.66 min. The rate constant is almost constant between pH 6 and 9 (τ_e = 180 min at pH = 7). At higher pH it decreases, with pK_a = 9.7 ± 0.2. At pH = 10.4 the lifetime of all-*trans*-bR is 20 h.

The blue ⇔ purple transitions at high pH

The main challenge in the present study was the detection of blue membrane at high pH, where its amount is very small.

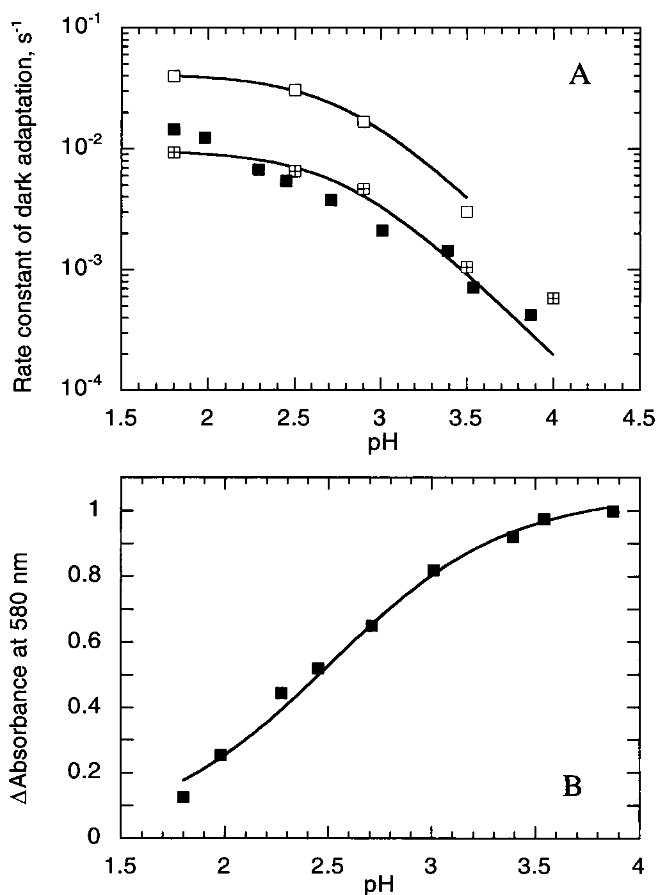

FIGURE 2 (*A*) pH dependence of the rate constant of the absorption change at 580 nm during dark adaptation of purple membrane at low pH. (□) Fast component in gels containing purple membrane that were light adapted at low pH. (⊠) Slow component in gels. (■) In suspensions of purple membrane that were light adapted at pH 7 and then adjusted to low pH in the dark. (*B*) Amplitude of the absorption change at 580 nm on dark adaptation in suspensions of purple membrane.

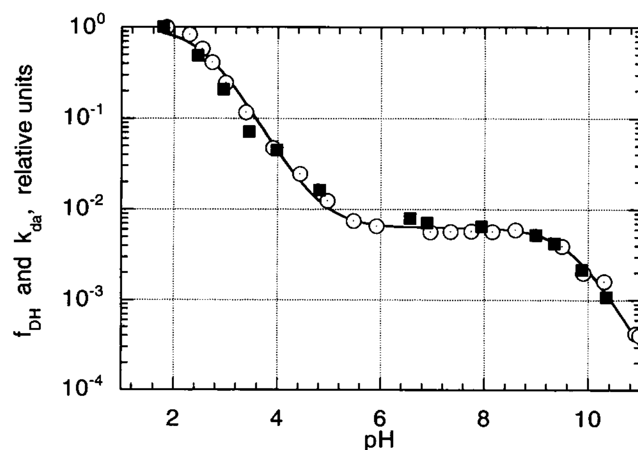

FIGURE 3 (■) pH dependence of the rate constant of dark adaptation, k_{da} (normalized to 1 at the maximum). The maximal rate of dark adaptation (at pH 1.8) was k^0_{da} = 0.01 s^{-1}. (○) Fraction of pigment having protonated Asp-85 (blue membrane), f_{DH}, in the dark-adapted bacteriorhodopsin calculated from the absorption changes at 690 nm (minus changes at 720 nm), which were taken from Fig. 4 below. The data were fitted by eq. 2 for pK_{a1} = 2.6, pK_{a2} = 9.7, and pK_{a3} = 4.8.

Assuming that the fraction of blue membrane can be described by the usual relationship for the fraction of protonated acid (Henderson–Hasselbalch equation; $f_{AH} = 1/(1 + 10^{(pH-pKa)})$; one can expect that the fraction of protonated Asp-85 at pH 6.6 and 9.6 will be equal to 10^{-4} and 10^{-7}, respectively (taking the pK_a for Asp-85 as 2.6 in the dark-adapted membranes). The accuracy of absorption measurements in the present study is ~ 10^{-4} OD units; thus no detectable changes in the fraction of blue membrane are expected at pH > 7 for a sample with OD = 1 of blue membrane at pH 2. However, if the hypothesis about proportionality of the rate constant of dark adaptation, k_{da}, and the fraction of protonated Asp-85, f_{DA} (Balashov et al., 1993), is correct, then from the pH dependence of k_{da} one would predict that the fraction of blue membrane should be ~ 170 times less at pH 7 than at pH 2 (that is, ~0.6% of the total pigment). For a sample of OD = 1 (at pH 2) at the monitoring wavelength (690 nm; see below), absorbance of the blue membrane would be ~6 mOD at pH 7. It should not change significantly between pH 5 and pH 9, but it should decrease by >90% as the pH is increased from 9 to 11. Changes of several mOD should be readily detectable.

A major complication in detection of the blue membrane at high pH is that two more transitions take place at pH > 8. They are the transformation of bR to an alkaline form, bR_a, which is accompanied by a small red shift of the absorption band, with pK_a = ~9.0, and transformation of bR into another alkaline species P480 (pK_a = 9.0 (6%) and pK_a = 11.8 (94%)), which causes a blue shift of the absorption band (Balashov et al., 1991). These transitions cause large absorbance changes in the purple membrane chromophore absorption band (400–680 nm), but they do not produce changes at longer wavelengths, where the absorbance of the blue membrane is still high. Thus, if absorbance changes of several mOD as a result of the blue-to-purple transition do take place at high pH, they could be detected by monitoring absorbance changes at 690–750 nm on increasing the pH.

Fig. 4 *A* shows a portion of the difference spectra obtained in a suspension of dark-adapted membranes as the pH is increased from 6.6 to 11.1. We suggest that the negative band seen in the spectra pH_i − pH 6.6 at 690–720 nm reflects the decrease in the amount of blue membrane as the pH is increased. The absorbance increase seen at shorter wavelengths is due to the red shift of the chromophore absorption band. The absorption changes at 690–720 nm produced by alkalinization are reversible: decreasing the pH from 11.1 to 6.6 causes an increase in absorbance at 690–720 nm, which can be interpreted as formation of blue membrane (Fig. 4 *A*, curve 8).

A plot of the absorption changes at 690–720 nm versus pH shows a transition with pK_a = 9.69 ± 0.06 (see Fig. 3 and Fig. 5 *A*). To minimize the contribution from changes of the baseline during titration, the difference in absorption between two wavelengths was taken (absorbance at 690 minus absorbance 720 nm). The absorbance of the blue membrane at 690 nm (minus the absorbance at 720 nm) at pH 6.6 was estimated to be 2.9 mOD from the fit of the pH-induced absorbance change at these wavelengths. Similar values of pK_a in the range 9.70 ± 0.15 were obtained for other pairs of wavelengths in this spectral region (685–720, 685–710, 690–725, 690–710, 695–720), indicating that these measurements give a reliable value for the pK_a of the blue-to-purple transition at high pH.

A plot of the data on a log scale (Fig. 5 *B*) reveals a remarkable similarity between the decrease of the rate constant of dark adaptation with pK_a = 9.7 and the absorption decrease at 690 nm that results from a decrease of the amount of blue membrane (see also Fig. 3).

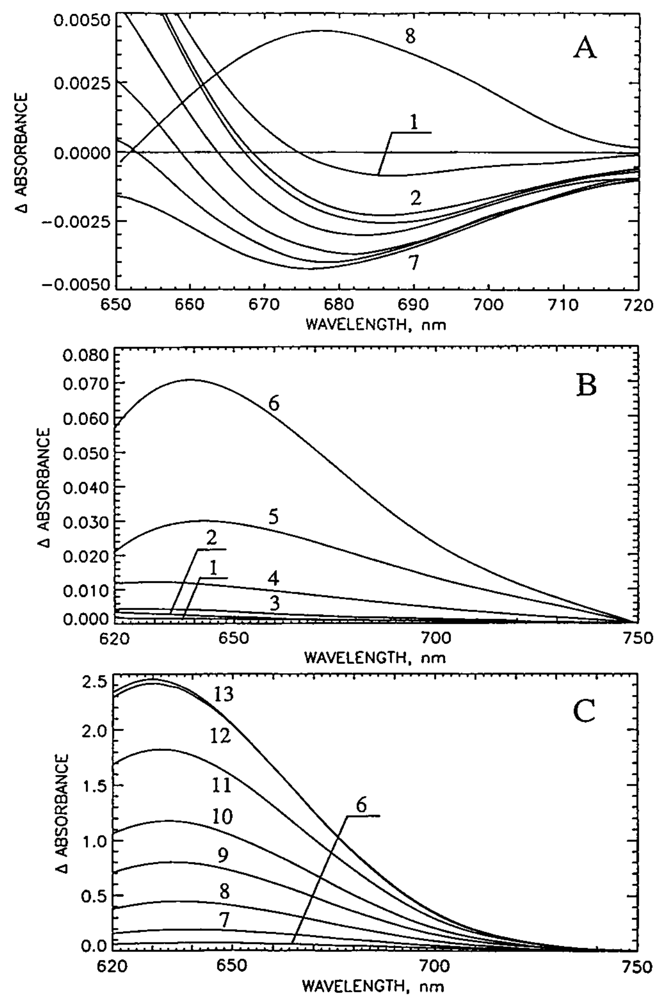

FIGURE 4 pH-induced absorption changes in a dark-adapted suspension of purple membrane in 150-mM KCl. (*A*) Curves 1–7, the difference spectra obtained on increasing the pH from 6.6 to 9.5, 9.9, 10.3, 10.7, 10.9, 11.0, and 11.1, respectively. The negative band at 720–690 nm is due to the blue-to-purple transition. At wavelengths shorter than 680 nm, this negative band overlaps the absorption changes with an opposite sign because of a 1-nm red shift of the chromophore's absorption band. Curve 8, Absorption changes on decreasing the pH from 11.1 to 6.6 (*B*), (*C*) Curves 1–13, difference spectra pH_i − pH 6.6, where pH_i is 6.2, 5.9, 5.5, 5.0, 4.4, 3.9, 3.4, 3.0, 2.7, 2.5, 2.3, 2.0, and 1.9. The optical density of the sample at the maximum (552 nm) was 4.5.

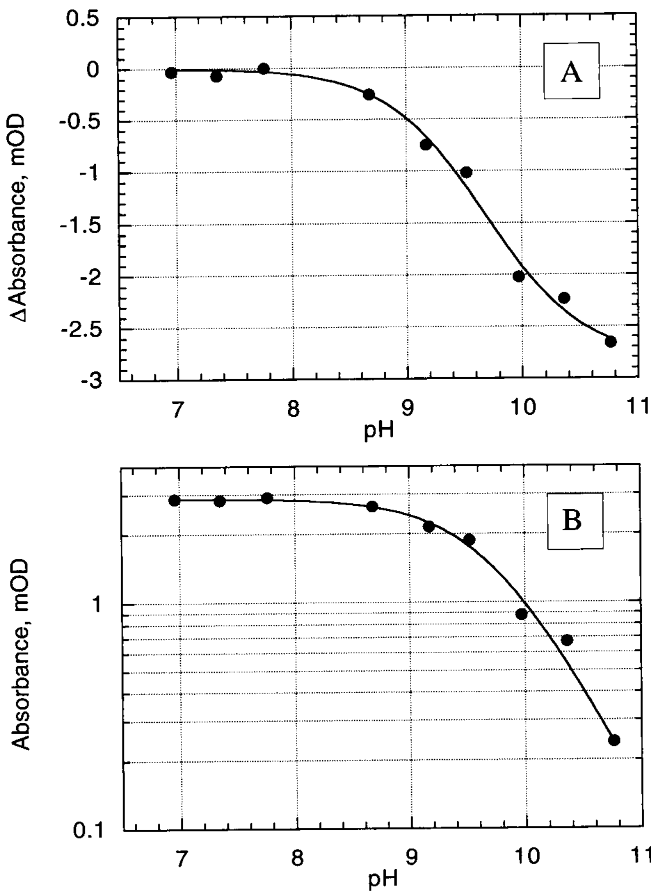

FIGURE 5 (*A*) pH dependence of absorption changes, ΔA, that are due to the blue-to-purple transition in dark-adapted bR. The points were taken from an experiment similar to that shown in Fig. 4 *A*. ΔA were obtained as a difference in absorbance changes at 690 and 720 nm, $\Delta A(\text{pH}) = \Delta A_{690}(\text{pH}) - \Delta A_{720}(\text{pH})$. A fit of the data with the Henderson–Hasselbalch equation, $\Delta A(\text{pH}) = -\Delta A_{max}/(1 + 10^{n(\text{pH}-\text{pK}_a)})$ (solid curve), indicates that a) $\text{pK}_a = 9.69 \pm 0.06$, b) deprotonation of only one residue takes place ($n = 1$), c) $\Delta A_{max} = -(2.9 \pm 0.1)$ mOD. (*B*) pH dependence of absorbance of the blue membrane in dark-adapted bR at 690 nm (minus absorbance at 720 nm). It was calculated as $A(\text{pH}) = 2.9 + \Delta A(\text{pH})$, assuming that absorbance of the blue membrane at pH 7 is 2.9 mOD. $\Delta A(\text{pH})$ were taken from (*A*). The optical density of the sample at the maximum was 4.5, as in Fig. 4.

Estimation of the fraction of blue membrane as a function of pH

To determine the fraction of blue membrane over a wide pH range we also titrated the suspension of membranes from pH 6.6 to pH 1.9. Figs. 4 *B* and *C* show the absorbance changes for a decrease in pH from 6.6 to 1.9. The plot indicates that the maximum difference between absorbance at 690 and 720 nm is ~650 mOD. Assuming that this corresponds to a complete transformation of the purple membrane into blue membrane, we can determine the fraction of blue membrane at any given pH. Thus at pH 7 it would be 2.9/650 = 0.45%, which is in good agreement with the prediction of the hypothesis. The fraction of blue membrane as a function of pH (f_{DH}) is shown in Fig. 3 (open circles). The shape of the curve is very similar to that of the pH dependence of the rate constant of dark adaptation, indicating that indeed the rate constant is proportional to the fraction of protonated Asp-85.

DISCUSSION

Proportionality between the rate constant of thermal isomerization and the fraction of protonated Asp-85; thermal isomerization occurs through the transient protonation of Asp-85

The remarkable similarity between the pH dependence of the rate constant of dark adaptation, k_{da}, and the fraction of protonated Asp-85, $f_{DH}(\text{pH})$, provides strong evidence that in WT bR, as in R82A (Balashov et al., 1993) and R82K (Balashov et al., 1995), the rate constant for dark adaptation is proportional to the fraction of the blue membrane (fraction of protonated Asp-85):

$$k_{da}(\text{pH}) = k^{\circ}_{da} f_{DH}(\text{pH}), \qquad (1)$$

where k°_{da} is the maximal rate constant of isomerization (observed when the fraction of protonated Asp-85 is close to 1).

This result implies that protonation of Asp-85 is the key (and rate limiting) factor in catalyzing thermal isomerization in bR and in determining the pH dependence of this process. We conclude that thermal isomerization, even at high pH, occurs via the blue membrane formed on transient protonation of Asp-85 (Fig. 6).

Thermal isomerization is at least 10^4 times less likely in the purple membrane than in the blue membrane (see Fig. 3). Protonation of Asp-85 reduces the barrier for isomerization, apparently because of increased delocalization of the π electrons in the chromophore (Warshel and Deakyne, 1978; Orlandi and Schulten, 1979; Sheves and Baasov, 1984; Tavan et al., 1985; Seltzer, 1990; Milder, 1991; Balashov et al., 1993, 1995; Song et al., 1993), and also probably by increasing the degree of freedom for the Schiff base motions because of the reduction of the electrostatic attraction between it and Asp-85 (Balashov et al., 1993), thus allowing the all-*trans* ⇔ 13-*cis* transition in the chromophore.

The above mechanism of isomerization may provide a possible explanation for the functional roles of the O intermediate and neutral Asp-85 in the late stages of the photocycle. The first part of the photocycle (from bR to M) results in the photoisomerization of the chromophore from all-*trans* to 13-*cis* and protonation of Asp-85. The N → O transition involves the 13-*cis* → *trans* thermal isomerization (Mathies et al., 1991), which we propose is catalyzed by the protonated state of Asp-85, and that is why Asp-85 stays protonated in the M, N, and O intermediates. Moreover, by analogy with the mechanism of dark adaptation one may expect that an O intermediate containing 13-*cis* chromophore should exist, as suggested by Milder (1991) and Ebrey (1993). During the photocycle the isomerization occurs only around the 13C═14C bond (Fodor et al., 1988)

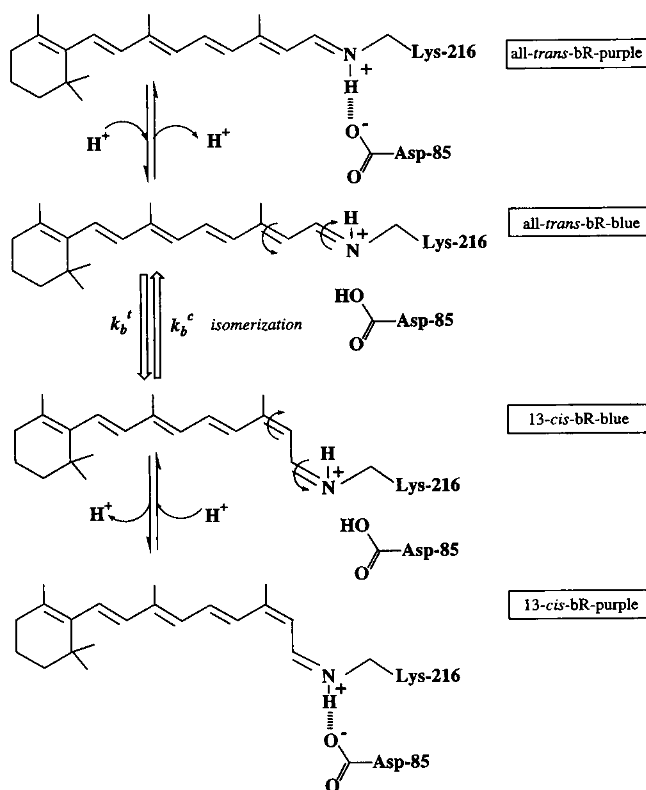

FIGURE 6 Schematic representation of the mechanism of thermal isomerization (dark adaptation) in bR. Transitions between all-*trans* and 13-*cis* isomers of bR occur through the protonation of Asp-85 (i.e., formation of blue membrane). k_b^t is the rate constant of all-*trans* → 13-*cis* isomerization of the chromophore of the blue membrane. k_b^c is the rate constant of the reverse process, 13-*cis* → all-*trans* isomerization in the blue membrane. The sum of k_b^t and k_b^c is equal to the maximum rate constant of dark adaptation, k°_{da}. The hatched lines (■) designate electrostatic interaction between Asp-85 and the protonated Schiff base. The structures of isomers are given according to Harbison et al. (1984) and Thompson et al. (1992), who showed that 13-*cis* and all-*trans*-bR differ not only in the configuration of the 13C=14C double bond but also in the configuration of the 15C=N bond (*anti* in all-*trans* and *syn* in 13-*cis*).

but not around the 15C=N bond, as in dark adaptation. However, the barrier for thermal isomerization should be lowered on protonation of Asp-85 during the photocycle also.

The catalytic role of protonation of Asp-85 does not exclude involvement of other mechanisms such as direct chromophore–protein interaction through the leucine-93 residue and C-13 methyl group of retinal (Subramaniam et al., 1991) in catalysis of thermal isomerization in bR.

Explanation of the complex titration curve of Asp-85; estimation of the values of pK_a of Asp-85 and X′

Interactions of amino acid residues in proteins may result in complicated titration curves for these groups. This was specifically proposed for Asp-212 and Asp-115 residues in bacteriorhodopsin based on theoretical calculations (Bashford and Gerwert, 1992; Sampogna and Honig, 1994).

To explain the pH dependence and the mechanism of thermal isomerization of the chromophore in bR we recently proposed a simple model that accounts for the complex titration behavior of Asp-85 (Balashov et al., 1993). We suggested that the pK_a of Asp-85 depends on the protonation state of another residue X′. When the residue X′ is protonated, the pK_a of Asp-85 is low (~2.6). When X′ deprotonates, the pK_a of Asp-85, increases by several pH units (Fig. 7). The pH dependence of the fraction of protonated Asp-85 in the framework of the model presented in Fig. 7 can be described as a function of three parameters (three values of pK_a) (Balashov et al., 1995):

$$f_{DH}(pH) = \alpha/(\alpha + \beta\gamma), \quad (2)$$

where $\alpha = 1 + 10^{(pH-pKa_3)}$, $\beta = 1 + 10^{(pH-pKa_2)}$, and $\gamma = 10^{(pH-pKa_1)}$ (see the legend to Fig. 7 for the definitions of the specific pK_a).

The fit of the experimental values of f_{DH} (Fig. 3) with Eq. 2 gives the following values: $pK_{a1} = 2.6$, $pK_{a2} = 9.7$, $pK_{a3} = 4.8$, and $pK_{a4} = 7.5$. Thus the fit of the experimental data with the model indicates that the pK_a of X′ is 9.7 and the pK_a of Asp-85 shifts from 2.6 to 7.5 on deprotonation of X′. The pK_a of X′ changes on protonation of Asp-85 from 9.7 to 4.8.

Implications for the proton release process

Effect of deprotonation of X′ on the proton transfer from the Schiff base to Asp-85

The increase in the pK_a of Asp-85 on deprotonation of X′ should stabilize the proton on Asp-85. This would decrease the rate of backreaction in the L ⇔ M transition (Zimányi et

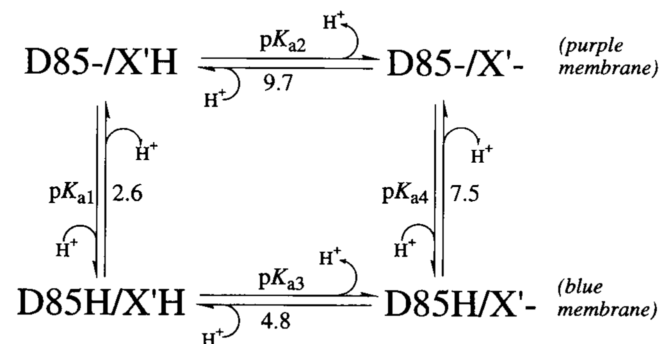

FIGURE 7 Scheme describing the equilibrium acid–base transitions of two interacting residues, Asp-85 and a residue (or group of residues) X′ that affects the pK_a of Asp-85. pK_{a1} is the pK_a of Asp-85 when X′ is protonated; pK_{a2} is the pK_a of X′ when Asp-85 is deprotonated; pK_{a3} is the pK_a of X′ when Asp-85 is protonated; pK_{a4} is the pK_a of Asp-85 when X′ is deprotonated. The four values of pK_a are not independent but rather are bound by a relationship: $pK_{a1} + pK_{a2} = pK_{a3} + pK_{a4}$. This relationship allows us to calculate an unknown pK_a from the three other known values for pK_a. The pK_a values were obtained from the fit of the experimental data of the fraction of protonated Asp-85, f_{DH}, shown in Fig. 3.

al., 1992; Balashov et al., 1995), thus facilitating the Schiff base deprotonation, especially at high pH.

The group X′ can act as a proton release group in the photocycle

Because the pK_a of the group X′ drastically decreases on protonation of Asp-85, it can act as a proton release group in the photocycle. That is, X′, the group controlling the pK_a of Asp-85, could be identified with X, the proton release group. Our estimate for the pK_a of X′ when Asp-85 is protonated in the ground (initial) state of bR is 4.8. In the M intermediate the proton release group has pK_a = 5.9, according to Zimányi et al. (1992). The difference between the pK_a of X′ in bR when Asp-85 is protonated (4.8) and the pK_a of the X group in M (5.9) can be attributed to the different protonation states of the Schiff base in bR and M.

Assuming that the group X′, which affects the pK_a of Asp-85, and the proton release group X is the same residue (or group of residues), one can predict certain features of the proton release process in bR. Depending on pH one can distinguish four cases (Fig. 8): 1) At high pH (pH > 9.7) most of X′ is already deprotonated in the ground state, and thus it cannot release a proton on the protonation of Asp-85 during M formation. This lack of fast light-induced proton release at high pH is in agreement with the decrease of the photocurrent associated with prompt proton release from purple membrane at high pH (Liu, 1990; Kono et al., 1993; Robertson and Lukashev, 1995). The proton release would occur presumably later in the photocycle, during the O-to-bR transition, perhaps directly from Asp-85. 2) Between pH 9 and pH 6 (in 150-mM KCl) the proton release group X is optimally functional; light causes it to deprotonate on approximately the same time scale as the M formation (Lozier et al., 1976; Drachev et al., 1984; Liu, 1990; Zimányi et al., 1992; Heberle et al., 1993; Alexiev et al., 1995). However, in some mutants the kinetic correlation of proton release and M formation is lost, even at neutral pH (Balashov et al., 1993; Heberle et al., 1993; Cao et al., 1995). 3) Between pH 5.9 (pK_a of X in M as estimated by Zimányi et al. (1992)) and 4.8 (pK_a of X′ when both the Schiff base and Asp-85 are protonated (Fig. 7) and so presumably close to its pK_a in N), most of the protons should be released from the X group during the M-to-N transition because reprotonation of the Schiff base during this transition should decrease the pK_a of X by ~ 1 pK unit, thus facilitating proton release. The proton release during the M-to-N transition is kinetically close to the proton uptake that follows this transition. The two proton flows cancel each other. This may explain the small amplitude of the transient proton signals detected in R82K mutant (Balashov et al., 1995). 4) At low pH (pH < pK_a of X in N) the proton release group is mostly protonated even after Asp-85 is protonated by light, and proton release will be delayed until deprotonation of Asp-85 during the O-to-bR transition (Cao et al., 1993). This happens after the proton uptake (Zimányi et al., 1992). Thus, depending on pH, proton release in bR is associated with one of the three photocycle reactions: a) L → M, b) M → N, c) O → bR.

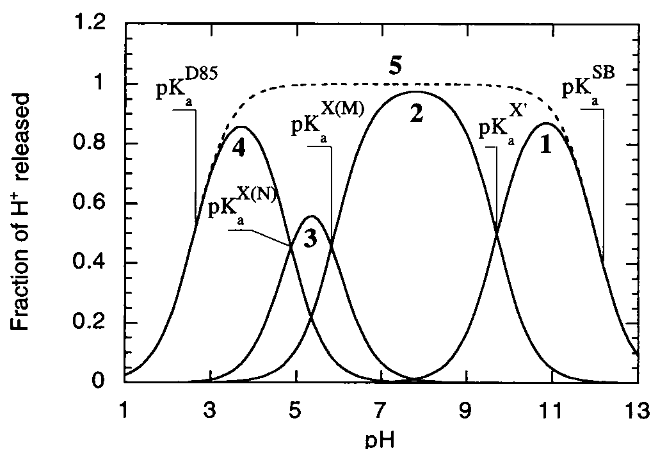

FIGURE 8 Schematic representation of the pH dependence for each of four different components of light-induced proton release by bR. 1, Possible proton release in the very high pH range where the normal proton release group is already deprotonated in the ground state. This would occur presumably not from the normal proton release group but through a different pathway, perhaps directly from Asp-85 during the O-to-bR transition. The pigment is supposed to be functional as long as the Schiff base is protonated in bR(pK_a^{SB} ~ 12.2). 2, Protons released from the proton release group during the L-to-M transition. The high-pH part of the curve decreases with pK_a of X′ (9.7), and the low pH part decreases with pH of X (5.9) as determined by Zimanyi et al. (1992). 3, Proton release in the pH range between pK_a of the proton release group in M (5.9) and pK_a in N where the Schiff base is reprotonated, which we tentatively assume to be 4.8 (see text). This component of proton release occurs from the proton release group X on approximately the time scale as that of the M-to-N transition. 4, Proton release in the pH range lower than the pK_a of proton release group X in N. It occurs presumably from Asp-85 during the O → bR transition. 5, The curve that represents functional activity of bR (fraction of purple membrane). At low pH it is determined by the purple-to-blue transition (pK_a^{D85} = 2.6) and at high pH by the transformation of bR into P480 species and eventually deprotonation of the Schiff base with pK_a = 12.2. All pK_a values are given for 150-mM KCl.

The identity of X′, the residue responsible for the change of pK_a of Asp-85

Based on several arguments, we proposed that Arg-82 is likely to be the group X′, which affects the pK_a of Asp-85 (or part of a complex of residues that constitute X′) and that Arg-82 may have a pK_a = 9.5–9.7 (Balashov et al., 1993, 1995). Other estimates by Brown et al. (1993) gave higher pK_a values for Arg-82 (pK_a = 13.8). If they are correct, the involvement of Arg-82 in the proton release is indirect, through its control of the pK_a of Asp-85 and the proton release group X rather than through direct deprotonation (Balashov et al., 1995). Theoretical calculations of Scharnagl et al. (1995) indicate that Glu-204 interacts with Arg-82 and may deprotonate on protonation of Asp-85. Earlier Drachev et al. (1993) also suggested that some carboxyl group with high pK_a is responsible for the changes in the rate constant of dark adaptation at high pH. Studies of E204 mutants can verify this possibility.

Residues in the counterion complex strongly interact with each other, and the pK_a of X′ is affected by R82A and R82K mutations (Balashov et al., 1993, 1995) and by Y57N and Y57F substitutions (Govindjee et al., 1995; Balashov et al., unpublished observation). The group X′ could in fact be a part of a cluster of hydrogen bonded residues including Asp-85, Arg-82, Tyr-57, Glu-204, and water molecules, which all contribute to the pK_a values of X′ (and X).

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