Evidence That Aspartate-85 Has a Higher pK_a in All-*trans* Than in 13-*cis*Bacteriorhodopsin

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ABSTRACT Three experimental observations indicate that the pK_a of the purple-to-blue transition (the pK_a of Asp-85) is higher for all-*trans*-bR[1] than for 13-*cis*-bR. First, light adaptation of bacteriorhodopsin (bR) at pHs near the pK_a of Asp-85 causes an increase in the fraction of the blue membrane present. This transformation is reversible in the dark. Second, the pK_a of the purple-to-blue transition in the dark is lower than that in the light-adapted bR (pK_a^{DA} = 3.5, pK_a^{LA} = 3.8 in 10 mM K_2SO_4). Third, the equilibrium fractions of 13-*cis* and all-*trans* isomers are pH dependent; the fraction of all-*trans*-bR increases upon formation of the blue membrane. Based on the conclusion that thermal all-*trans* ⇔ 13-*cis* isomerization occurs in the blue membrane rather than in the purple, we have developed a simple model that accounts for all three observations. From the fit of experimental data we estimate that the pK_a of Asp-85 in 13-*cis*-bR is 0.5 ± 0.1 pK_a unit less than the pK_a of all-*trans*-bR. Thus, in 10 mM K_2SO_4, pK_a^c = 3.3, whereas pK_a^t = 3.8.

INTRODUCTION

Bacteriorhodopsin (bR), the light-driven proton pump of the purple membrane (Oesterhelt and Stoeckenius, 1971), exists in the dark as a mixture of two forms, 13-*cis*-bR and all-*trans*-bR, which have 13-*cis*,15-*syn* retinal and all-*trans*,15-*anti* retinal as their chromophores, respectively (Oesterhelt et al., 1973; Harbison et al., 1984; Thompson et al., 1992). Upon illumination (light adaptation) 13-*cis*-bR is transformed into all-*trans*-bR. The latter can undergo a cyclic photoreaction that results in transmembrane proton transfer. The process involves photoisomerization around the 13C=14C double bond, conformational changes of the protein, transient protonation/deprotonation of several amino acid residues, and thermal reisomerization of the chromophore (Birge, 1990; Henderson et al., 1990; Mathies et al., 1991; Rothschild, 1992; Ebrey, 1993; Lanyi, 1993).

Decreasing the pH causes the so-called purple-to-blue spectral transition (Fischer and Oesterhelt, 1979; Mowery et al., 1979; Kimura et al., 1984; Chang et al., 1985; Jonas and Ebrey, 1991), leading to a complete loss of functional activity of the pigment. The purple-to-blue transition is primarily due to the protonation of the Schiff base counterion, Asp-85 (Subramaniam et al., 1990; Metz et al., 1992a).

Studies of the wild-type bR and R82 mutants of bacteriorhodopsin in which positively charged arginine-82 was replaced by alanine or lysine showed an almost perfect correlation between the purple-to-blue transition and the pH dependence of dark adaptation (thermal isomerization) (Ohno et al., 1977; Warshel and Ottolenghi, 1979; Balashov et al., 1993, 1995, 1996). We proposed that dark adaptation is catalyzed by the transient protonation of Asp-85, which dramatically decreases the barrier for thermal isomerization (Balashov et al., 1993). The direct proportionality between the fraction of protonated Asp-85 (the fraction of blue membrane) and the rate of thermal all-*trans* ⇔ 13-*cis*-isomerization was established for the R82A and R82K mutants (Balashov et al., 1993, 1995) as well as for the wild type (Balashov et al., 1996) over a wide range of pH. The proportionality requires that isomerization occurs in the blue membrane (upon protonation of Asp-85) rather than in the purple membrane, even at high pH.

In this paper we studied the processes of light and dark adaptation of native (wild-type) purple membrane at pHs close to the pK_a of the purple-to-blue transition. We found that illumination of the dark-adapted (DA) membranes at these pHs results in a partial purple-to-blue transition, which reverses in the dark. This suggests that all-*trans*-bR, which is produced by light adaptation, has a higher pK_a for the purple-to-blue transition (pK_a of Asp-85) than 13-*cis*-bR. To describe the processes of interaction of light and dark adaptation with the purple-to-blue transition, we developed a simple model which indicates that different pK_as for the purple-to-blue transition in all-*trans*-bR and 13-*cis*-bR explain the pH dependence in the fractions of these isomers at pHs near the pK_a of this transition.

MATERIALS AND METHODS

Purple membrane was isolated from the *Halobacterium salinarium* strain S9 according to the method of Becher and Cassim (1975). To prevent aggregation at low pH the purple membranes were incorporated into polyacrylamide gels as described previously (Liu et al., 1991). The gels

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Abbreviations: bR, bacteriorhodopsin; 13-*cis*-bR and all-*trans*-bR, ground state forms of bacteriorhodopsin having 13-*cis*, 15-*syn* retinal and all-*trans*, 15-*anti* retinal as their chromophores, respectively; DA, dark-adapted; LA, light-adapted; LI, light-induced; CCCP, carbonyl cyanide m-chlorophenyl-hydrazone.

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were equilibrated at a given pH by incubation in buffer solutions for about 12 h. A different gel slice was used for each pH. pK_a of protonation of Asp-85 and of the purple-to-blue transition depend on salt concentration (Jonas and Ebrey, 1991); another transition at low pH (blue shift) depends on the presence of chloride ions (Fischer and Oesterhelt, 1979). To check salt and ion dependence of the light-induced changes in the fraction of the blue membrane, experiments were done using two different salt solutions, 10 mM K_2SO_4 and 150 mM KCl. The pH was set between 1.5 and 5 with 10 mM citric acid, adjusted by the addition of H_2SO_4 (or HCl) and KOH. The absorption spectra and kinetics of dark adaptation were recorded on a Cary-Aviv model 14DS UV-VIS spectrophotometer. All measurements were made in a thermostatted 5-mm cuvette at 20°C. Light adaptation was produced with a 500-W slide projector with $CuSO_4$ solution and cutoff filters to isolate the actinic light (400–550 nm) and prevent heating of the sample during illumination. This light did not cause formation of the pink species, which contains the 9-*cis* isomer of retinal (Maeda and Yoshizawa, 1980; Fischer et al., 1981; Chang et al., 1987). For excitation of the blue species only, illumination at $\lambda > 660$ nm was used.

RESULTS

The light-induced purple-to-blue transition in bR at low pH

Dark-adapted bacteriorhodopsin between pH 5 and 2 is a mixture of two forms, purple membrane and blue membrane. Illumination of this mixture at 400–550 nm at these pHs results in an increase in the fraction of blue membrane. Fig. 1 *A* shows changes in the difference spectra accompanying light adaptation of the bR-containing gels at pHs from 7.2 to 2.7 (in 10 mM K_2SO_4). At pH 7.2 the usual difference spectrum, peculiar to the transformation of the purple form of 13-*cis*-bR to all-*trans*-bR (Oesterhelt et al., 1973), is observed. The main maximum in the difference spectrum of light-adapted (LA) minus DA membranes is at 588 nm, the minimum is at 500 nm, and isosbestic point is at 530 nm. The absorption changes at wavelengths longer than 670 nm are negligible. At shorter wavelengths there are several sharp maxima (so called β-bands) at 428, 398, 372, 350, 331, and 318 nm. At pH 4.7 the difference spectrum is very close to that at pH 7.2; however, there is some absorbance increase at 670–700 nm. At pH 4.1 the maximum in the spectrum decreases in amplitude and shifts to the red, a substantial increase in absorption in the far red region (670–750 nm) is observed, and the amplitudes of the β-bands greatly decrease. At pH 3.3, the maximum is at 632 nm and minimum is at 540 nm, with an isosbestic point at around 575 nm. At lower pH (pH 2.7) some additional shift of the isosbestic point to 585 nm is observed, and the amplitude of the difference spectrum decreases.

The blue species produced by light adaptation is spectrally similar to that produced by a decrease in pH, as shown in Fig. 1 *B*. The difference spectrum of the absorption changes produced by illumination at 400–550 nm at pH 3.6 is almost identical to that for the purple-to-blue transition, produced by changing the pH from 7.2 to 3.6 (Fig. 1 *B*). Thus the blue membrane can be produced not only by acid (Oesterhelt and Stoeckenius, 1971) or removal of cations (Kimura et al., 1984; Chang et al., 1985), but also by illumination at certain pHs.

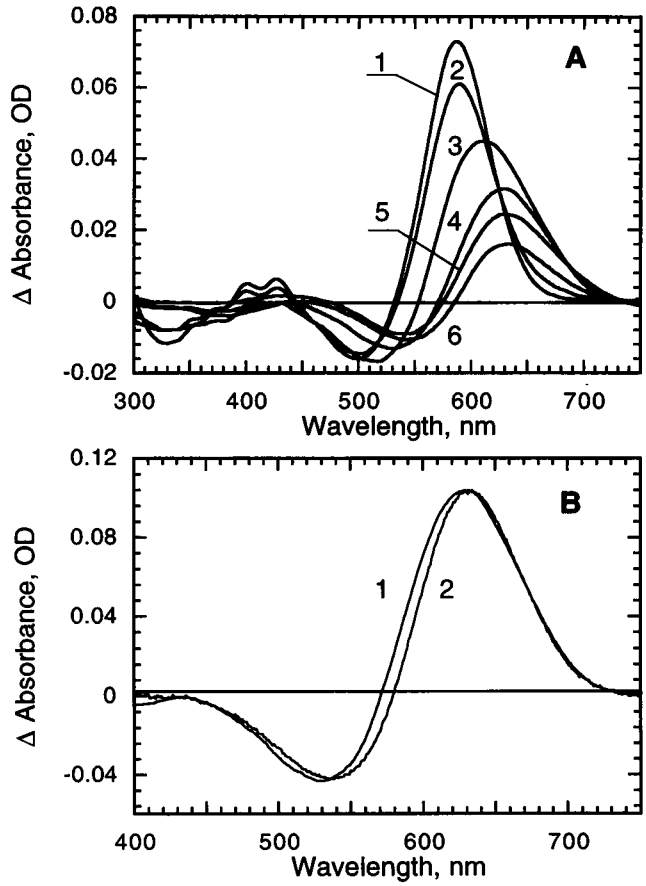


FIGURE 1 Formation of additional blue membrane by the illumination of bR at low pH. (*A*) Curves 1 through 6, difference absorption spectra produced by 400–550-nm illumination of dark-adapted purple membrane incorporated in gels and equilibrated at pH 7.2, 4.7, 4.1, 3.6, 3.3, or 2.7, respectively. (*B*) Absorption changes produced by 400–550-nm illumination of the dark-adapted purple membrane at pH 3.6 (1); decreasing the pH from 7.2 to 3.6 in the dark (2). Spectrum 1 was multiplied by 3.29 to normalize it with spectrum 2.

The spectra in Fig. 1 *A* indicate that light adaptation at pHs between 5 and 2 causes two processes: the transformation of 13-*cis*-bR-purple into all-*trans*-bR-purple and the purple-to-blue transition. The contribution of the first process can be estimated from the light-induced absorbance changes at 580 nm (a wavelength close to the isosbestic point of the purple-to-blue transition) (Fig. 2 *A*). The amplitude of the absorption changes at 580 nm decreases with the decreasing pH (pK_a = 3.8 in 10 mM K_2SO_4, and pK_a = 2.9 in 150 mM KCl). This decrease is presumably due to the decrease in the fraction of purple-bR at low pH.

The amount of the blue species produced by light was estimated from the absorption changes at 630 nm or 660 nm, after subtraction of the absorbance changes associated with the transformation of 13-*cis*-bR-purple into all-*trans*-bR-purple. At 630 nm the contribution of this process is substantial (34% of the absorbance change at 580 nm); at 660 nm the contribution is small (6% of the change at 580 nm),

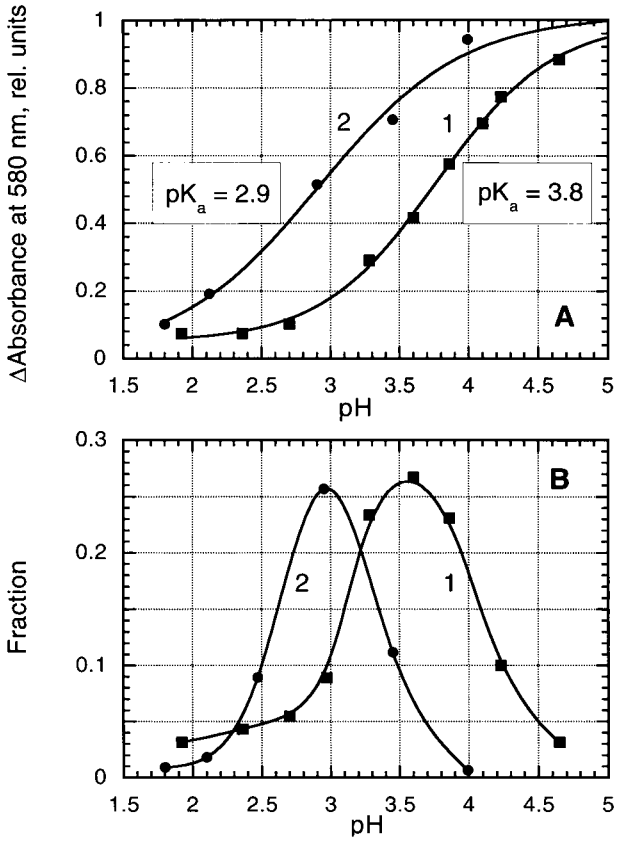


FIGURE 2 pH dependence of the additional blue membrane formed by illumination. (*A*) pH dependence of the amplitude of absorption changes at 580 nm associated with the transformation of 13-*cis*-bR-purple into all-*trans*-bR-purple under illumination at 400–550 nm (light adaptation of the purple forms of bR): 1, in 10 mM K_2SO_4; 2, in 150 mM KCl. Absorption changes at pH 7.2 were normalized to 1. Curves are fits with the Henderson-Hasselbalch equation. (*B*) Fraction of the pigment that undergoes the light-induced purple-to-blue transition: 1, in 10 mM K_2SO_4; 2, in 150 mM KCl. The fractions were estimated from the absorption changes at 660 nm in 10 mM K_2SO_4, and at 630 nm in 150 mM KCl. The absorption changes were measured after switching off the actinic illumination (which initiates the transition from the dark-adapted to the light-adapted state). The light-induced absorbance changes at 630 nm, ΔA_{630}, include two components. One is due to the light adaptation of the purple species, ΔA^{P}_{630}, and the other is due to the light-induced formation of the blue membrane, ΔA^{B}_{630}. From spectrum 1 in Fig. 1 *A*, where only the first process occurs, it follows that ΔA^{P}_{630} is equal to 0.34 of absorption changes at 580 nm. The values of ΔA^{B}_{630} were obtained as a difference: $\Delta A^{B}_{630} = \Delta A_{630} - \gamma\Delta A_{580}$, where $\gamma = 0.34$. Similar absorption changes at 660 nm were deconvoluted using $\gamma = 0.06$. The fractions were calculated by dividing the light-induced absorption changes ΔA^{B}_{630} by the acid-induced absorption changes corresponding to presumably complete transition of purple species into the blue species measured at pH 2.3 in 10 mM K_2SO_4 (Fig. 3) and at pH 1.5 in 150 mM KCl.

and the absorption changes are mainly due to formation of blue membrane. As shown in Fig. 2 *B*, the amount of light-induced blue species reaches its maximum at a pH close to the pK_a of the purple-to-blue transition. At both salt concentrations examined, a maximum of about 26% of the total pigment undergoes the light-induced purple-to-blue transition.

Illumination shifts the pK_a of the purple-to-blue transition in bR

The larger amount of the blue species observed after illumination suggests that the pK_a of the purple-to-blue transition is higher after light adaptation than in the dark. The pK_a of the purple-to-blue transition in DA membrane can be obtained from the spectral changes in the DA gels produced by decreasing the pH in the dark; the changes in 10 mM K_2SO_4 are shown in Fig. 3 *A*. The difference spectra have an isosbestic point at 580 nm and a maximum at 630 nm (Fig. 3 *B*). The absorption changes at 660 nm versus pH are plotted in Fig. 4. They can be fitted by the Henderson-Hasselbalch equation giving the pK_a for the DA membrane at 3.50 ± 0.05 (n = 1.2 ± 0.1). In 150 mM KCl, pK_a = 2.59 ± 0.02, n = 1.5 ± 0.1.

To estimate the pK_a of the purple-to-blue transition in the LA membranes we added the light-induced absorption changes at 660 nm (see Fig. 2 *B*) to the absorption changes produced by acidification of the DA membrane. The pK_a plot for the purple-to-blue transition in the light is shown in Fig. 4. In 10 mM K_2SO_4 the pK_a = 3.80 ± 0.05, n = 1.4 ± 0.1 (in 150 mM pK_a = 2.90 ± 0.05, n =

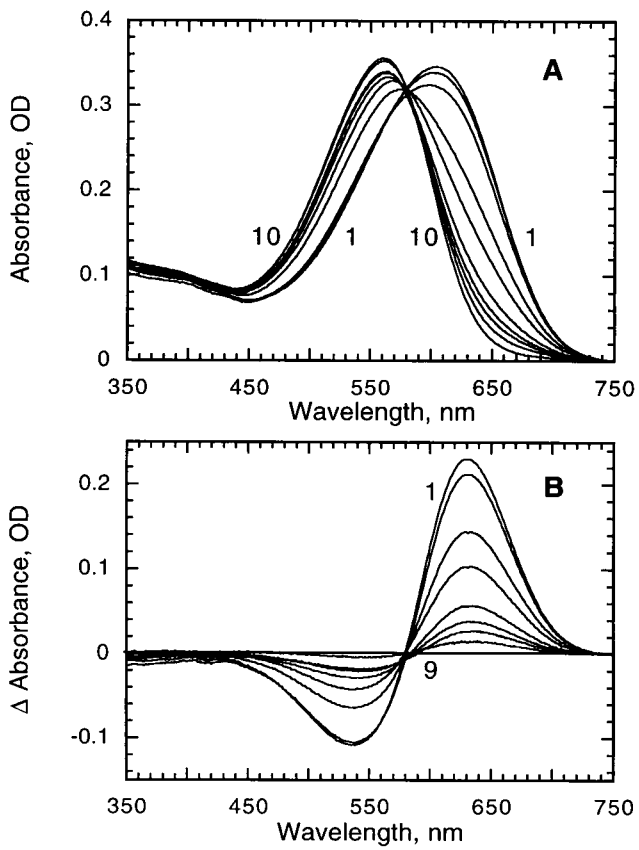


FIGURE 3 pH dependence of the absorption spectra of dark-adapted purple membrane in 10 mM K_2SO_4. (*A*) 1 through 10, pH_i equal to 2.4, 2.7, 3.0, 3.3, 3.6, 3.9, 4.1, 4.2, 4.7, and 7.2, respectively. (*B*) 1 through 9, the difference absorption spectra obtained as spectrum at pH_i minus that at pH 7.2.

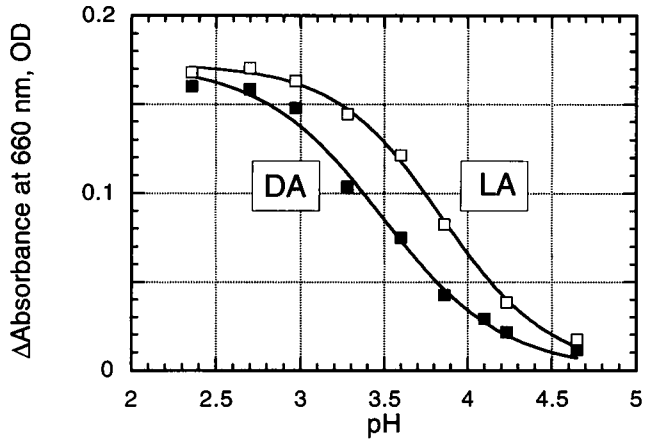


FIGURE 4 pH titration of the purple-to-blue transition in dark-adapted (DA) and in light-adapted (LA) gels measured as absorption changes at 660 nm. DA data (■) are taken from the difference spectra shown in Fig. 3 *B*. LA absorption changes (□) at 660 nm obtained as a sum of absorption changes in the dark plus absorption changes produced by illumination (10 mM K_2SO_4). Curves are the fits with $pK_a = 3.5$, $n = 1.2$ for DA membranes; $pK_a = 3.85$, $n = 1.35$ for LA membranes.

1.1 ± 0.1). Thus the pK_a in the LA gels is shifted about 0.3 pH units to higher pH compared to that for the DA gels. This is in agreement with the observation of Mowery et al. (1979) that there is a larger amount of the blue species in LA membranes than in DA membranes at around pH 3.

Reformation of the initial purple state during dark adaptation at low pH

The absorption changes produced by 400–550 nm illumination are reversible in the dark. A set of spectra measured at pH 3.6 (in 10 mM K_2SO_4) after switching off the light are given in Fig. 5, *A* and *B*. They indicate that the all-*trans*-bR-purple and all-*trans*-bR-blue produced by illumination are transformed back to the initial DA state. It is interesting that the shape of the spectra does not change significantly with time. This suggests that both transitions (blue-to-purple and all-*trans*-purple to 13-*cis*-purple) proceed in parallel, with only one being rate limiting.

The half-time of relaxation of the light-induced blue species is about 2 min at pH 3.6 and 25 s at pH 3.0 in 10 mM K_2SO_4. Between pH 3 and pH 4.5 the kinetics are described by a sum of at least three exponential components with pH-independent rate constants. The fast relaxation component has a lifetime of 30 s ($k = 0.03\ s^{-1}$). The second and third components have lifetimes of about 2 min ($k = 0.007\ s^{-1}$) and 8 min ($k = 0.002\ s^{-1}$), respectively. At pH 3.6 the amplitudes of the kinetic components represent almost equal fractions (0.33). At lower pH, contributions of the slow components decrease, and below pH 3 only the fast kinetic component is observed.

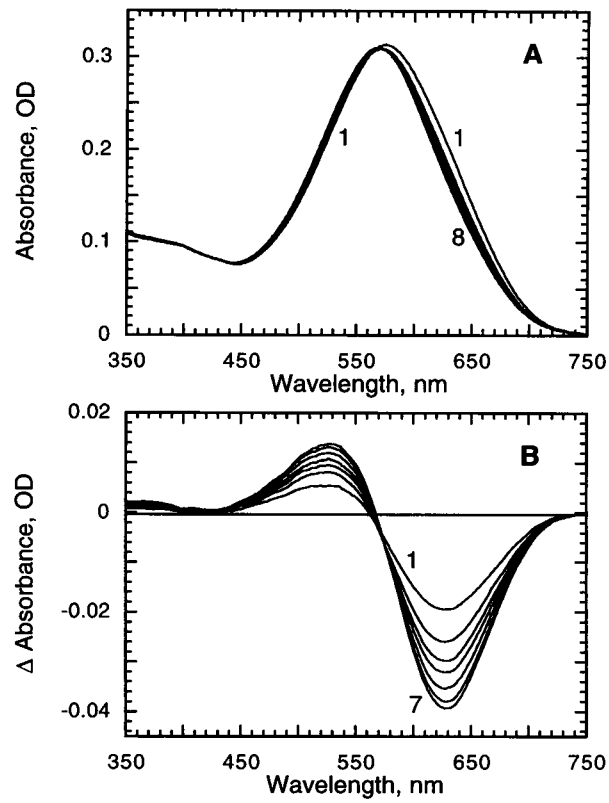


FIGURE 5 Absorption changes during dark adaptation at pH 3.6. (*A*) Spectra 1 through 8 recorded 12 s and 5, 10, 15, 20, 30, 45, and 60 min after the end of illumination (10 min at 400–550 nm). (*B*) The difference spectra 1 through 7 are the differences between spectrum 1, taken 22 s after illumination, and the spectra taken after 5, 10, 15, 20, 30, 45, and 60 min of incubation in the dark at 20°C. The time to record one spectrum is 3 min (10 mM K_2SO_4).

The blue-to-purple photoconversion induced by red light illumination

Illumination of the DA membranes at pH 2.0 with red light ($\lambda > 660$ nm) causes a partial transformation of the blue species into the species absorbing at shorter wavelengths (Fig. 6 *A*, *curve 1*). The new species is the purple membrane, because the difference spectrum is the same as that of the purple-to-blue transition (taken with the opposite sign). In the dark the reverse process takes place: the purple species reverts back to the blue (see the series of spectra shown in Fig. 6 *B*). The difference spectrum of this transition is very close to that observed after illumination with blue light (Fig. 5 *B*), but the sign is opposite. From these data we conclude that light can cause not only the purple-to-blue but also blue-to-purple transition. The net direction of the photoreaction depends on the wavelength of excitation. Blue light induces mainly the purple-to-blue transition, whereas red light drives the opposite reaction. Red light illumination also causes the blue-to-pink transition (formation of 9-*cis*-pigment absorbing around 490 nm; Chang et al., 1987), which is not reversible in the dark (at least on an hour time scale) (Fig. 6 *A*, *curve 3*).

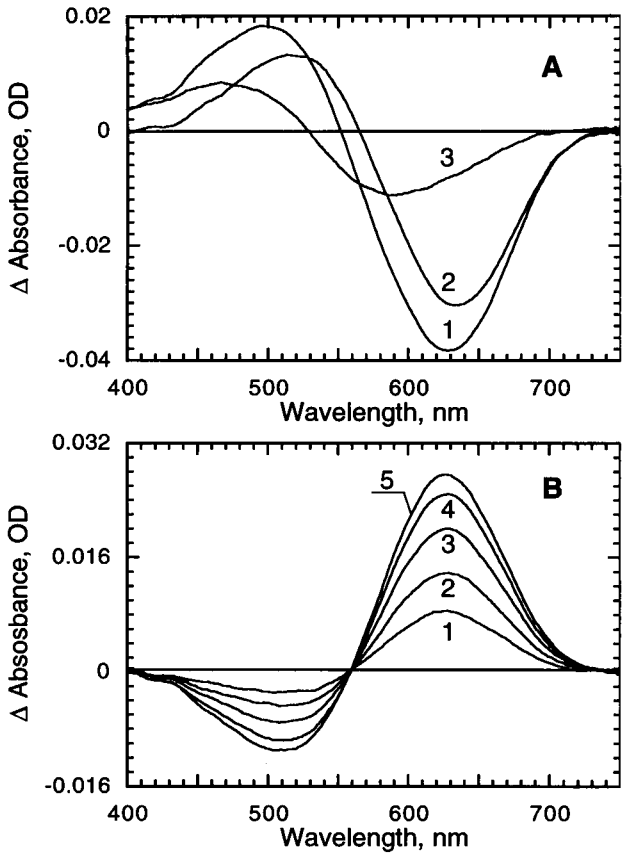


FIGURE 6 Blue-to-purple photoconversion induced by red light illumination. (*A*) 1, absorption changes produced by 10 min of illumination of dark-adapted membranes at pH 2 with red light ($\lambda > 660$ nm); 2, component due to the blue-to-purple transition, which reversed after 1 h of incubation in the dark; 3, component due to the blue-to-pink transition, which remained after 1 h of incubation in the dark. (*B*) Purple-to-blue transition in the dark after 10 min of illumination at $\lambda > 660$ nm: 1–5, spectra obtained as a difference between spectra recorded 6, 12, 24, 48, and 96 min in the dark minus spectrum recorded 30 s after illumination.

pH dependence of the all-*trans*/13-*cis* isomer ratio in bacteriorhodopsin

Several reports have concluded that at low pH the fraction of all-*trans*-bR increases (Fischer and Oesterhelt, 1979; Mowery et al., 1979; Pande et al., 1985; Chang et al., 1987; Kaulen and Postanogova, 1990). This phenomenon can be explained by a simple model (see Discussion), assuming that the pK_a of the purple-to-blue transition is different in all-*trans*-bR and 13-*cis*-bR and that isomerization takes place in the blue membrane form of the pigment. In this model the pH dependence of the fractions of the isomers can be used as an independent method to determine the ΔpK_a between the two isomers (see Eq. 3 below). To prove the applicability of the model to the explanation of the pH dependence of the isomer ratio, it was important to demonstrate that changes in the equilibrium fraction of all-*trans* and 13-*cis* isomers are indeed coupled to the purple-to-blue transition .

Fig. 7 shows the measurements used to obtain the fraction of all-*trans*-bR as a function of pH. The fraction of all-*trans*-bR was determined from the amplitude of the β-band at 428 nm, which is present in the spectrum of all-*trans* but not 13-*cis*-bR (Litvin and Balashov, 1977). To increase resolution of this band, we measured the amplitude of its second derivative (Fig. 7 *A*), in a manner similar to that used to resolve mixtures of all-*trans*-bR and its primary batho-product *K* at low temperatures (Balashov et al., 1991). At pH 7 the amplitude of this band in the dark-adapted sample is only 0.35 of that in the light-adapted one (Fig. 7 *A*). The corresponding β-band of 13-*cis*-bR is blue-shifted compared to the 428-nm band of all-*trans*-bR and does not contribute significantly to its amplitude in the second derivative spectrum (Fig. 7 *B*). Assuming that in light-adapted bR 100% of the pigment is in the all-*trans* configuration, we conclude that in the dark-adapted membrane the fraction of all-*trans*-bR is 35–36%. This value is close to the value of 33–35% found by Scherrer et al. (1987) and others (see Discussion). However, several other estimations have found a higher value, ~45% (Fischer and Oesterhelt, 1979; Tsuda et al., 1980; Kaulen and Postanogova, 1990). The exact value for the amount of 13-*cis*-bR in DA membrane was not crucial for this study. The main goal was to check if an increase in the fraction of all-*trans*-bR occurs with the same pK_a as the purple-to-blue transition.

The pH dependence of the fraction of all-*trans*-isomer was determined in the following way. A suspension of purple membrane was incubated in the dark at a given pH_i (pH between 6 and 2). At pH between 2 and 3.5 a 1-h incubation was used. At pH between 4 and 6, the sample was incubated overnight at 20°C to achieve full equilibrium between isomers. The pH of the sample was then adjusted to pH 7 in the dark at 15°C, and the absorption spectrum was measured immediately after. The second spectrum was taken after a 24-h incubation for complete dark adaptation. The difference between these two spectra corresponds to the excess of all-*trans*-bR in the DA membranes at pH_i compared to the equilibrium amount at pH 7 (we assume that during the change in pH from pH_i to pH 7 the isomer fractions do not change significantly). The amplitude of the β-bands (Fig. 7 *C*) was used as a measure of the fraction of all-*trans*-bR. Assuming that the LA minus DA difference spectrum corresponds to the transformation of all the 13-*cis* (65% of the total pigment) into all-*trans*-bR, the relative amplitudes of the difference spectra were converted into absolute fractions, shown in Fig. 7 *D*. Thus, in the sample that was equilibrated at pH 2 and then brought to pH 7 in the dark, the fraction of all-*trans*-bR is 24% larger than the equilibrium concentration at pH 7, so the total concentration of all-*trans*-bR at pH 2 is 59%. Similar measurements of samples that were equilibrated at different pHs and then brought to pH 7 resulted in the estimation of equilibrium fraction of all-*trans*-bR at different pHs, shown in Fig. 7 *D*. The pK_a of the increase in the fraction of all-*trans*-bR was the same ($pK_a = 3.5$; $n = 1$) as the pK_a of the purple-to-blue transition in this sample, which confirms that the two transitions have a common origin (protonation of Asp-85).

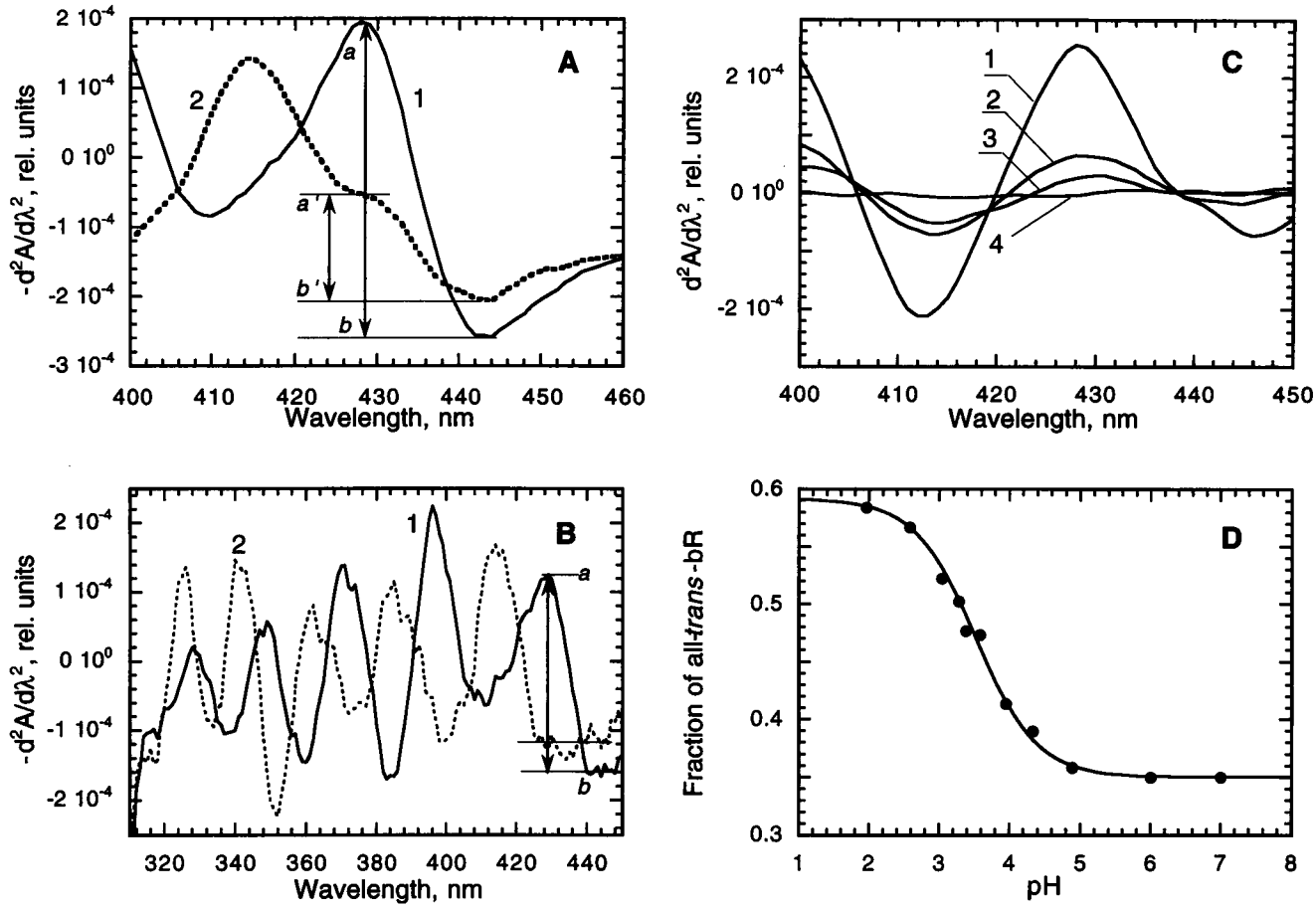


FIGURE 7 pH dependence of the all-*trans*/13-*cis* isomer ratio. (*A*) Estimation of the fraction of all-*trans*-bR in the dark-adapted purple membrane at pH 7 from the amplitude of the 428-nm band. To reveal the band, a second derivative of absorption spectra of light-adapted (1) and dark-adapted (2) membrane was taken. The fraction of all-*trans*-bR was determined as a ratio of the amplitudes of the 428-nm band in DA and LA samples (*a'b'/ab*). (*B*) Second derivatives of the β-bands of all-*trans* (1) and 13-*cis*-bR (2). Spectrum 2 was calculated from the spectrum of DA and LA membranes, assuming that LA contains 100% all-*trans*-bR and DA contains 65% 13-*cis*-bR. (*C*) Estimation of the fraction of all-*trans*-bR from the amplitude of the 428-nm band in the second derivative of the difference spectra, all-*trans* minus 13-*cis*-bR. 1, Difference spectrum light-adapted minus dark adapted at pH 7; 2, 3, and 4, difference spectra dark-adapted at pH_i and measured at pH 7 minus spectra dark-adapted at pH 7, where pH_i is 2.7, 3.5, and 4.9 for curves 2, 3, and 4, respectively (see details in the text). (*D*) Fraction of all-*trans*-bR in the dark-adapted membranes in 10 mM K_2SO_4. ●, Experimental data; curve, fit with Eq. 3a for the case $pK_a^t = 3.8$, $pK_a^c = 3.3$, and $K = 0.7$.

DISCUSSION

The light-induced purple-to-blue transition and pK_a of Asp-85

The data presented indicate that at $2 < pH < 5$, illumination of the DA membranes with 400–550-nm light results in an increase in the fraction of the blue membrane. The simplest explanation for this is that irradiation converts some 13-*cis*-bR to all-*trans*-bR, and the all-*trans*-bR has a higher pK_a for the purple-to-blue transition than the 13-*cis*-bR, resulting in the formation of additional blue membrane:

$$\text{13-}cis\text{-bR-purple} \xrightarrow{h\nu} \text{all-}trans\text{-bR-purple} \longrightarrow \text{all-}trans\text{-bR-blue.}$$

Because protonation of Asp-85 is responsible for the purple-to-blue transition, our finding suggests that the pK_a of Asp-85 is higher in all-*trans*-bR than in 13-*cis*-bR.

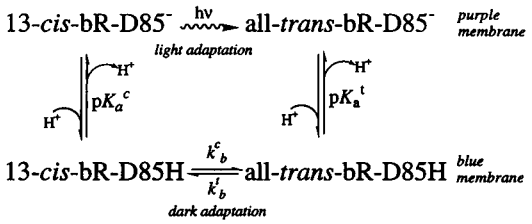
An alternative explanation for the observed light-induced absorbance changes is that they are due to the transformation of the low-pH form of 13-*cis*-bR (13-*cis*-bR-acid) into all-*trans*-bR-blue. According to Ohtani et al. (1986), the low-pH acid form of 13-*cis*-bR absorbs at 560 nm, whereas the acid form of all-*trans*-bR absorbs at 605 nm. This reaction, however, cannot be an important contribution to the observed effect, because in this case the transition should be a maximum at $pH < 2$, when the concentration of the acid form of 13-*cis*-bR would be a maximum. Contrary to this prediction, the effect we observe is very small at $pH < 2$, but is a maximum near the pK_a of the purple-to-blue transition, in agreement with the first mechanism.

Another alternative explanation is based on the observation of Nasuda-Kouyama et al. (1990) of the light-induced formation of blue-bR in suspensions of vesicles containing bacteriorhodopsin at pHs close to the pK_a of

the purple-to-blue transition. In this closed membrane system the spectral change is caused by a light-induced decrease of pH on the N-terminal side of the purple membrane, due to transmembrane proton transfer to the inside of the vesicle. This effect does not play a major role in our case, because the purple membrane exists as open sheets in which a pH gradient cannot be formed. Moreover, the light-induced purple-to-blue transition we observed was not affected by a high concentration of buffer or a protonophore (20 mM sodium azide, or 10^{-5} M carbonyl cyanide *m*-chlorophenyl-hydrazone; data not shown), giving additional evidence that the spectral change is not connected with the formation of a light-induced pH gradient or increase in the local H^+ concentration on the surface of the membrane.

Effect of chromophore isomerization on pK$_a$ of Asp-85: a model to estimate the pK$_a$ of the purple-to-blue transition in all-*trans*-bR and 13-*cis*-bR

The pK$_a$ of the purple-to-blue transition of all-*trans*-bR, pK$_a^t$, should be very close to the pK$_a$ for the LA purple membrane (because most or all of the LA pigment is in the all-*trans* configuration). The pK$_a$ of 13-*cis*-bR, pK$_a^c$, can be determined from the model given below, which accounts for the light-induced formation of the blue species and pH dependence of isomer fractions. The blue membrane contains both all-*trans* and 13-*cis* pigments (Fisher and Oesterhelt, 1979; Mowery et al., 1979; Pande et al., 1985; Smith and Mathies, 1985), which are apparently in equilibrium with each other and the purple species. We propose that the following cycle takes place during the light-induced purple-to-blue transition and reverse transformation in the dark:



Illumination of bacteriorhodopsin transforms 13-*cis*-bR-purple into all-*trans*-bR-purple. All-*trans*-bR-purple converts partially into all-*trans*-bR-blue. The pK$_a$ of the purple-to-blue transition in all-*trans*-bR is higher than in 13-*cis*-bR, pK$_a^t$ > pK$_a^c$, because the DA titration curve is shifted to lower pHs (Fig. 4). In the dark, relaxation of the system from the nonequilibrium state produced by light takes place. All-*trans*-bR thermally isomerizes into 13-*cis*-bR with a rate constant k_b^t Isomerization takes place in the blue state, because the rate of isomerization in the blue membrane (where Asp-85 is protonated) is at least $5 \cdot 10^3$ times larger than in the purple (Balashov et al., 1993, 1996). 13-*cis*-bR-blue in the dark equilibrates with 13-*cis*-bR-purple.

At equilibrium in the dark, the rate of isomerization from all-*trans*-bR-blue to 13-*cis*-bR-blue is equal to the rate of the reverse reaction. Assuming that the rates of isomerization in both directions are proportional to the fractions of species in which Asp-85 is protonated, a simple relationship can be written:

$$k_b^t f_t f_{D85H^t} = k_b^c f_c f_{D85H^c}, \quad (1)$$

where f_t and f_c are the fractions of species having all-*trans* and 13-*cis* chromophores, respectively ($f_t + f_c = 1$); n, the number of protons taken up in the purple-to-blue transition, is assumed equal to 1; k_b^t and k_b^c are the rate constants of isomerization of all-*trans* and 13-*cis* pigments in the blue membrane, respectively. For the case when the fraction of blue membrane can be described by a simple Henderson-Hasselbalch relationship,

$$f_{D85H^t} = 1/(1 + 10^{(pH - pK_a^t)}), \quad f_{D85H^c} = 1/(1 + 10^{(pH - pK_a^c)}), \quad (1a)$$

Equation 1 transforms into

$$k_b^t f_t/(1 + 10^{n(pH - pK_a^t)}) = k_b^c f_c/(1 + 10^{n(pH - pK_a^c)}). \quad (1b)$$

Three experimental observations can be explained by the model in which the pK$_a$ of the purple-to-blue transition is higher in all-*trans*-bR than in 13-*cis*-bR ($\Delta pK_a = pK_a^t - pK_a^c \neq 0$). These are different pK$_a$s of the purple-to-blue transition in DA and LA membranes, the pH dependence of isomer fractions, and light-induced formation of blue membrane. Using the available data and the equations derived from the model, we can estimate the values for pK$_a^t$, pK$_a^c$, and $K = k_b^t/k_b^c$.

The calculated titration curves for the purple-to-blue transitions in DA and LA membranes are different (Fig. 8,

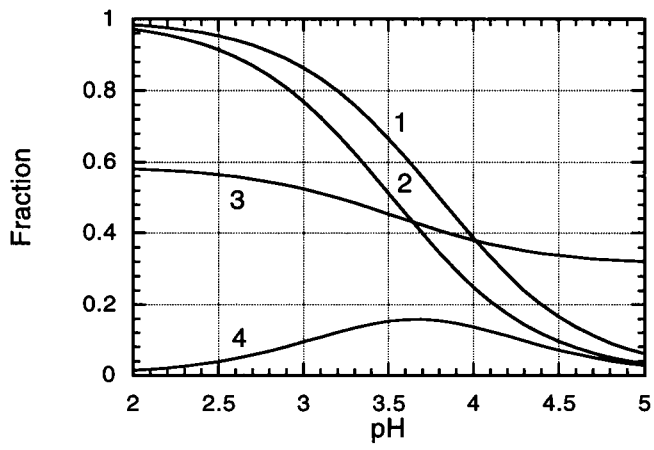


FIGURE 8 (*A*) Analytical curves for the wild type. 1, Fraction of blue species in light-adapted membrane; 2, fraction of blue species in dark-adapted membrane; 3, fraction of all-*trans*-bR in the dark-adapted membrane; 4, fraction of light-induced blue membrane. Curves 2, 3, and 4 were calculated from Eqs. 2a, 3a, and 4a, respectively, for pK$_a^t$ = 3.8; pK$_a^c$ = 3.3, K = 0.7. The pK$_a$ in DA membrane is 3.5.

curves 1 and 2, and Eq. 2a, below). The light-induced shift in the pK_a between LA and DA membranes, $\Delta pK_a^{LI} = pK_a^{LA} - pK_a^{DA}$, depends upon pK_a^t, pK_a^c, and K. The fraction of the blue species in LA membranes can be obtained from the Henderson-Hasselbalch equation $f_b^{LA} = 1/(1 + 10^{(pH - pK_a^t)})$ (assuming that only the all-*trans* isomer is present in LA membranes). The fraction of the blue species (sum of all-*trans*-bR-blue and 13-*cis*-bR-blue) in the DA membranes, f_b^{DA}, is given by the following function of pH:

$$f_b^{DA} = (1 + K)[(f_{D85H^t})^{-1} + K(f_{D85H^c})^{-1}]^{-1}. \quad (2)$$

For the case described by Eq. 1a, this transforms into

$$f_b^{DA} = (1 + K)[(1 + 10^{(pH-pK_at)}) + K(1 + 10^{(pH-pK_ac)})]^{-1}. \quad (2a)$$

Taking $pK_a^t = 3.8$ (Fig. 2 *A*) and varying the values for pK_a^c and K, we tried to fit the curve of the purple-to-blue transition in the dark with $pK_a^{DA} = 3.5$, shown in Fig. 4. The analytical curve of f_b^{DA} for the case $pK_a^t = 3.8$, $pK_a^c = 3.3$, and $K = 0.70$ (Fig. 8, *curve 2*) has a pK_a of 3.5. However, the choice of pK_a for 13-*cis*-bR is not unique. For example, a similar curve can be obtained with very different parameters ($pK_a^c = 2.5$ and $K = 0.05$). To narrow the possible range of solutions some other data must be taken into consideration. An additional source of information on the pK_a of 13-*cis*-bR is the pH dependence of isomer fractions and the fraction of light-induced blue bR.

The pH dependence of the equilibrium fraction of the all-*trans*-isomer, f_t (sum of the fraction of all-*trans*-blue and all-*trans*-purple), can be calculated as

$$f_t = [1 + Kf_{D85H^t}/f_{D85H^c}]^{-1}. \quad (3)$$

For the case of Eq. 1a, Eq. 3 results in

$$f_t = [1 + K(1 + 10^{(pH-pK_ac)})(1 + 10^{(pH-pK_at)})]^{-1}. \quad (3a)$$

The pH dependence of f_t (Fig. 8, *curve 3*) has the same pK_a as the purple-to-blue transition in the dark, pK_a^{DA}, which means that even though all-*trans*-bR and 13-*cis*-bR may have different pK_as of the purple-to-blue transition, under equilibrium conditions the fractions of all-*trans*-bR-blue and 13-*cis*-bR-blue would change with the same pK_a^{DA}.

In the blue membrane the fraction of all-*trans*-bR f_t is equal to $1/(1 + K)$, and in the purple, to $1/(1 + K \times 10^{\Delta pKa})$. Thus from the values of isomer fractions in the purple and blue membrane the value of ΔpK_a and the rate constant ratio, K, can be determined. In most studies the fraction of all-*trans*-bR at neutral pH was found to be close to 45–50% (Oesterhelt et al., 1973; Sperling et al., 1977; Fischer and Oesterhelt, 1979; Tsuda et al., 1980; Kaulen and Postanogova, 1990; Schulte et al., 1995). However, a significantly lower value, 33–38% (Scherrer et al., 1987; Ihara et al., 1994; Turner et al., 1993; Song et al., 1995), has also been reported. At pH 0, where the acid purple form is mainly present, the fraction of all-*trans*-bR increases to about 83–90% (Fischer and Oesterhelt, 1979; Mowery et al., 1979). In the blue membrane (at pH around 2) the fraction of all-*trans*-bR increases to 70% (Fischer and Oesterhelt, 1979; Pande et al., 1985; Kaulen and Postanogova, 1990). According to Smith and Mathies (1985) the ratio of all-*trans*/13-*cis* isomer is 60% to 40% in the DA blue membrane, and according to de Groot et al. (1990) it is 55% to 45%, whereas at neutral pH it is the opposite, 45% to 55%. Despite the scatter, all data indicate some (10–30%) increase in the fraction of all-*trans*-bR in the DA blue membrane (at pH 2) compared to the DA purple membrane at neutral pH.

Our data obtained from the analysis of the amplitude of the β-bands show 35% all-*trans* at pH 7 and 59% all-*trans*-bR after a jump from pH 2 to 7 (Fig. 7 *D*). The fit of these data with Eq. 3a gave the following estimates: $\Delta pK_a = pK_a^t - pK_a^c = 0.43$ and $K = 0.7$. The $K = 0.7$ implies that in the blue membrane the rate constant of thermal isomerization of 13-*cis* chromophore is at least 1.5 times faster than the rate of isomerization of the all-*trans*-chromophore. The fit of the data of Kaulen and Postanogova (1990) (45% all-*trans* in the blue membrane and 70% in the purple) with Eq. 3 gave $\Delta pK_a = 0.5$ and $K = 0.4$. Thus the difference in the equilibrium fractions of all-*trans*-bR obtained in the latter and present studies mainly affects the value of K.

Modeling of the light-induced purple-to-blue transition

Illumination of DA membrane changes the fraction of the blue membrane (Fig. 2 *B*). The fraction of blue bR formed upon illumination of DA membrane as a function of pH can be calculated as a difference between the amount of blue membrane in LA and DA membranes:

$$f_b^{LI} = f_b^{LA} - f_b^{DA}. \quad (4)$$

For the case described by Eq. 1a this transforms into

$$f_b^{LI} = K(10^{(pH-pK_ac)} - 10^{(pH-pK_at)})/ \quad (4a)$$
$$[(1 + 10^{(pH-pK_at)})(1 + 10^{(pH-pK_at)} + K(1 + 10^{(pH-pK_ac)})].$$

The experimental data (Fig. 2 *B*) are in general agreement with the theoretical curve given by Eq. 4a (Fig. 8, *curve 4*). The experimental value (26%) is slightly larger than predicted by the model (about 20%). The source for this discrepancy is not clear; it is most likely associated with $n > 1$ for the experimental titration curves.

On the nature of the pK_a difference in all-*trans* and 13-*cis*-bR

The data presented above indicate that the pK_a of Asp-85 in 13-*cis*-bR is approximately 0.5 pH units lower than that in all-*trans*-bR. A difference in the pK_as could also be inferred from ^{13}C solid-state NMR data, which showed that the chemical shifts of deprotonated Asp-85 and Asp-212 are sensitive to light and dark adaptation (Metz et al., 1992b). At present one may only speculate about the cause of different pK_as of Asp-85 in all-*trans* and 13-*cis*-bR. A

reasonable explanation would be different electrostatic interaction energies between the protonated Schiff base and the negatively charged Asp-85, due to slightly different distances between them in all-*trans*-bR and 13-*cis*-bR. The decrease in the distance, Δr, required to produce a decrease in pK_a by 0.5 units depends strongly on the effective dielectric constant, ϵ, used in the calculations. Using the point charge approximation, Δr is estimated to be 0.7 Å if $\epsilon = 15$ (as proposed by Balashov et al. (1993) for the Asp-85-Arg-82 electrostatic interaction), and $\Delta r = 0.2$ Å if $\epsilon = 4$ (following Sharp and Honig, 1990). In these estimations the distance between the Schiff base and the carboxyl oxygen of Asp-85 was assumed to be 4.2 Å (unpublished data of R. Henderson, cited in Song et al., 1993). An alternative explanation is that the different geometries between the Schiff base linkage and Asp-85 in the two isomers induce a different hydrogen bonding pattern between these two groups, involving the water molecules that form a bridge between them (Gat and Sheves, 1993). This can be interpreted as a change in the effective dielectric constant.

In the photocycle, the chromophore is isomerized from all-*trans* to 13-*cis*,15-*anti* (Mathies et al., 1991; Fodor et al., 1988). It is evidently associated with a much larger perturbation of the Schiff base–Asp-85 interaction than during dark adaptation, where additional isomerization around the 15C=N bond takes place. Photoisomerization in the photocycle results in the destabilization of this ion pair, transfer of proton to Asp-85, and a dramatic increase in the pK_a of Asp-85 in M (to more than 10) due to a change in the environment of Asp-85 (Braiman et al., 1996).

Generalization of the model for the pH dependence of the two isomer forms of bR to account for the complex titration behavior of Asp-85: comparison of light-induced purple-to-blue transition in the wild type (WT) and mutants of bR

Equations 2a and 3a were derived under the assumption that the titration of Asp85 can be described by a simple Henderson-Hasselbalch equation (1a). However, this is a simplification that may not hold in some cases. The pK_a of Asp-85 is strongly coupled to the protonation state of some other ionizable residue, X′, which most likely acts as the proton release group during the photocycle (Balashov et al., 1993, 1996). The deprotonation of X′ increases the pK_a of Asp-85 by several pH units (~4.7 ± 0.2 units in the WT). As a result, the titration curve of the blue membrane (in which Asp-85 is protonated) shows a second transition, with pK_a corresponding to deprotonation of X′ (Balashov et al., 1995, 1996). Theoretical calculations of Scharnagl et al. (1994) and experimental data of Brown et al. (1995) and Richter et al. (1996) suggest that Glu-204 functions as the proton release group and is X′, the residue that controls the pK_a of Asp-85.

In the WT, the fraction of blue membrane is small at neutral pH (less than 1% of total pigment) (Balashov et al., 1996), and Eq. 1a is quite sufficient for the above analysis. However, in some mutants, like D85E, the fraction of blue membrane is very high (about 75%) at pH between 5 and 9. Titration of blue membrane in D85E shows two clear transitions with pK_a 4.6 and 9.5, which has been interpreted as being due to some kind of pigment heterogeneity (Lanyi et al., 1992). Wild-type bR also shows the two pK_a titration behavior, which was explained by the four-state model of two interacting residues (Balashov et al., 1993, 1996). In the framework of this model, the increased amount of the blue membrane in D85E can be explained by an increase in the second pK_a of Glu-85 (the pK_a when X′ is deprotonated); when this pK_a becomes close to the pK_a of X′, the fraction of blue membrane becomes large, even at high pH (Balashov et al., 1995). One may expect additional formation of blue membrane during light adaptation if it results in an increase in the fraction of all-*trans* isomer.

An interesting situation has been described for the Tyr185→Phe mutant of bR (Y185F), in which light adaptation results in the formation of a large amount of a red-shifted species at pH < 10 (Dunach et al., 1990; Sonar et al., 1993; Rath et al., 1993; He et al., 1993). It was formed during light adaptation along with the usual all-*trans*-bR purple species and decayed in parallel with it. This red-shifted species was proposed to be an O-like intermediate, in equilibrium with the purple form of all-*trans*-bR or, alternatively, a blue form (blue membrane) of the all-*trans*-isomer of Y185F.

The latter case can be simulated using the four-state diagram for the description of the fraction of blue membrane (Fig. 9 and Balashov et al., 1993, 1996), assuming that the pK_a of Asp-85 is about 0.5–1 pH unit higher in all-*trans*-Y185F-bR than in 13-*cis*-Y185F-bR. Because the pK_a of Asp 85 depends on the chromophore configuration, we should consider two different pK_a sets for the 13-*cis*-bR and all-*trans*-bR. All four pK_a values may be sensitive to the chromophore configuration. For the case shown in the diagram, we assumed that the pK_a of group X′ was the same for both all-*trans* and 13-*cis* pigments (equal to 8.7), whereas the pK_a of Asp-85 was 0.7 pH units higher in *trans*-bR than in 13-*cis*-bR (the pK_as for the 13-*cis* isomer

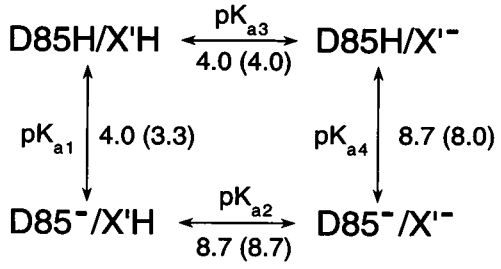


FIGURE 9 Four-state diagram showing coupling of the pK_a of Asp-85 to the protonation state of another group X′. pK_a values are chosen to qualitatively simulate the properties of Y185F mutant described by Sonar et al. (1993). Numbers without parenthesis correspond to the all-*trans*-bR; numbers in parenthesis correspond to the 13-*cis*-bR. See text for details.

are given in parentheses). The pK_a of Asp-85 in the DA Y187F mutant is increased to 3.6 from 2.6 in the WT (Sonar et al., 1993). We assumed that deprotonation of X′ increased the pK_a of Asp-85 by 4.7 pH units (similar to that in the WT).

The fraction of protonated Asp-85 in *trans*-bR, f_{D85H^t}, and in 13-*cis*-bR, f_{D85H}, can be calculated as functions of three pK_as:

$$f_{D85H^t} = \alpha^t/(\alpha^t + \beta^t\gamma^t); \quad f_{D85H^c} = \alpha^c/(\alpha^c + \beta^c\gamma^c); \quad (5)$$

where $\alpha = 1 + 10^{(pH - pK_{a3})}$; $\beta = 1 + 10^{(pH - pK_{a2})}$; and $\gamma = 10^{(pH - pK_{a1})}$ (see Balashov et al., 1993, 1996); the superscripts t and c refer to the pigment having all-*trans* or 13-*cis* chromophores. The pK_as are shown in the diagrams for all-*trans* and 13-*cis*-bR. Equation 5 is a more general case that should be used instead of Eq. 1a at pHs where the fraction of blue membrane is large.

According to the concept of coupling of Asp-85 and X′ (Balashov et al., 1993, 1996), deprotonation of X′ increases the pK_a of Asp-85 from pK_{a1} to pK_{a4}. Correspondingly, protonation of Asp-85 reduces the pK_a of X′ from pK_{a2} to pK_{a3}. In the WT, these changes in the pK_as are quite large, ~4.7 ± 0.2 pK_a units (Balashov et al., 1993, 1995).

Fig. 10 shows the fractions of protonated Asp-85 in the LA and DA states and light-induced changes in the fraction of blue membrane as a function of pH for the case described by the diagram in Fig. 9. The most interesting feature is that the light-induced increase in the fraction of the red-shifted species is observed over a wide range of pH (between pK_{a1} and pK_{a2}). Thus the model describes the features that were observed in the Y185F mutant (Sonar et al., 1993) and may be useful for estimating the pK_as of Asp-85 and the group X′ in this and many other mutants that show increased amounts of blue membrane at neutral pH (because the values of pK_{a2} and pK_{a3} are close). It predicts that the fraction of all-*trans*-bR in the DA Y185F should have a second inflection point (Fig. 10, *curve 4*). The present model based on the four-state diagram does not require the pK_a of Asp-85 in all-*trans*-bR to be very different from that of 13-*cis*-bR. One pH unit difference is enough to account for the observed effects. The above described model supports the possibility that in the Y185F mutant the light-induced red-shifted photoproduct (Dunach et al., 1990; Sonar et al., 1993) is the blue membrane; however, other possibilities should be investigated. The interesting peculiarity of Y185F mutant is that the light-induced red-shifted species has a very long lifetime in this mutant, presumably because of its very slow rate of dark adaptation (Sonar et al., 1993), which indicates that Tyr-185 may play a role in thermal isomerization of the chromophore. An alternative explanation is a very slow rate of deprotonation of Asp-85.

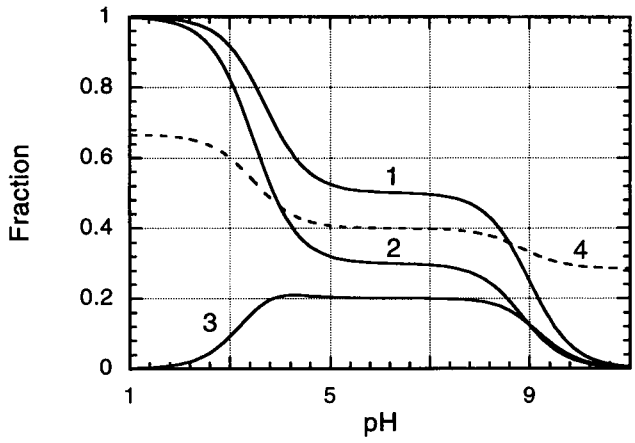


FIGURE 10 Analytical curves for the case when the amount of blue membrane is high at neutral pH and the titration of Asp-85 shows two transitions (at low and high pH). 1, Fraction of blue species (with protonated Asp-85) in the light-adapted membrane (assuming that it contains 100% all-*trans*-bR); 2, fraction of blue species in the dark-adapted membrane (calculated using Eqs. 2 and 5); 3, light-induced increase in the fraction of blue membrane, obtained as a difference between curves 1 and 2; 4, fraction of all-*trans* isomer in the dark-adapted state (calculated using Eqs. 3 and 5). The following parameters were used: $pK^t_{a1} = 4.0$, $pK^c_{a1} = 3.3$, $pK_{a2} = 8.7$, $pK_{a3} = 4.0$ (both for 13-*cis* and *trans* isomers); $K = 0.5$.

The blue-to-purple transition induced by red light illumination

To explain the blue-to-purple transition produced by red light illumination, we suggest that red light causes photoisomerization of the blue form of all-*trans*-bR into the blue form of 13-*cis*-bR:

$$\text{all-}trans\text{-bR-blue} \xrightarrow{h\nu} 13\text{-}cis\text{-bR-blue}.$$

Because 13-*cis*-bR-blue has a lower pK_a for the purple-to-blue transition, part of the pigment will then thermally convert into the purple form of 13-*cis*-bR. In the dark the reverse processes take place. Normally photoconversion from all-*trans*-bR into 13-*cis*-bR is prohibited in bR. However, under dehydrating conditions in dry films (Kouyama et al., 1985) or at high concentrations of glycerol (Balashov et al., 1988), light causes photoisomerization in both directions, from 13-*cis* to all-*trans* and from all-*trans* to 13-*cis*-bR. Apparently a water molecule near the Schiff base prevents photoisomerization around the 15C=N bond (Balashov et al., 1988). Removal of this molecule results in the possibility of photoisomerization in both directions. A similar effect is caused by the protonation of Asp-85. We assume that protonation of Asp-85 causes changes in the environment of the Schiff base and decreases the barrier for photoisomerization around the 15C=N bond.

CONCLUSION

Light induces the transformation of the purple form of bR into the blue form at pHs close to the pK_a of the purple-to-blue transition. This effect is presumably due to the higher pK_a of the purple-to-blue transition (pK_a of Asp-85) in all-*trans*-bR than in 13-*cis*-bR. A model based on this assumption is developed that accounts for the light-induced

purple-to-blue transition and pH dependence of the isomer ratio in bR at low pH. From the fit of the data it follows that the pK_a of the purple-to-blue transition in all-*trans*-bR is about 0.5 pH units higher than in 13-*cis*-bR. The rate constant of thermal isomerization from 13-*cis* to all-*trans*-bR in the blue membrane is 1.5 times larger than the opposite process. It is found that the red light illumination of the blue form causes a blue-to-purple transition.

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