The pH Dependence of the Subpicosecond Retinal Photoisomerization Process in Bacteriorhodopsin: Evidence for Parallel Photocycles

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ABSTRACT The pH dependence of the subpicosecond decay of the retinal photoexcited state in bacteriorhodopsin (bR) is determined in the pH range 6.8–11.3. A rapid change in the decay rate of the retinal photoexcited state is observed in the pH range 9–10, the same pH range in which a rapid change in the M_{412} formation kinetics was observed. This observation supports the previously proposed heterogeneity model in which parallel photocycles contribute to the observed pH dependence of the M_{412} formation kinetics in bR.

INTRODUCTION

Bacteriorhodopsin (bR) is the only protein in the purple membrane of *Halobacterium halobium* (Oesterhelt and Stoeckenius, 1971). The physiological role of bR is to translocate protons electrogenically across the purple membrane. This vectorial proton translocation across the membrane generates a pH gradient that is used for adenosine triphosphate (ATP) synthesis. Upon the absorption of visible light, the energy stored in bR drives the following photocycle consisting of intermediates that are formed on time scales ranging from subpicosecond to milliseconds:

$$bR_{568} \xrightarrow{h\nu} (bR)^* \xrightarrow{0.2\ ps} I_{460} \xrightarrow{0.5\ ps} J_{625}$$

$$\xrightarrow{\sim 3\ ps} K_{610} \xrightarrow{2\ \mu s} L_{550} \underset{-H^+}{\xrightarrow{70\ \mu s}} M_{412} \underset{+H^+}{\xrightarrow{ms}} N \rightarrow O \rightarrow bR_{568}$$

In the photocycle described above, M_{412} is the only intermediate in which the protonated Schiff base (PSB) is deprotonated. Transient optical measurements (Hanamoto et al., 1984; Dupuis et al., 1985; Diller et al., 1988; Bitting et al., 1990; Varo et al., 1990; Lin et al., 1991; Balashov et al., 1991; Fukuda et al., 1992) and photoelectric measurements (Ormos et al., 1985; Liu et al., 1990) have revealed that the M_{412} formation is at least biphasic with rate constants differing by roughly an order of magnitude. The relative amplitude of the slow component of M_{412} formation showed a titration type dependence with rapid change in the pH range 8–12 (Lin et al., 1991). The rate constant of the slow component of M_{412} formation was found to be pH-independent between pH 3 and 7 but increases continuously above pH 8 (Hanamoto et al., 1984; Lin et al., 1991).

The origin of the biphasic nature of the M_{412} formation is not quite clear at present. There are basically two models to explain it. In the first model, the heterogeneity model, which was first proposed by Hanamoto et al. in 1984 and supported by other results (Diller et al., 1988; Dancshazy et al., 1988; Balashov et al., 1991; Komrakov et al., 1994), explains the biphasic nature by two (or more) parallel photocycles resulting from having the protein Schiff base in two (or more) different environments, most likely resulting from the acid-base equilibria that exist for one or more of the many amino acid residues in bR.

In the other model (Ames et al., 1990; Varo et al., 1990; Druckman et al., 1992), one photocycle with pH-dependent reverse (back) reactions were used to fit the observed kinetics at high pH. In this model, as the pH increases, the rates of the M→L and N→M back reactions change, resulting in an apparent biphasic kinetics for the formation and decay of the M_{412} intermediate, that is pH-dependent.

The back reaction is a diffusion-controlled process and occurs on the microsecond to millisecond time scale. It is not expected to affect the kinetics of the rapid decay of the photoexcited state of retinal occurring on the subpicosecond time scale. According to the one photocycle-back reaction model, the rate of photoisomerization should be pH-independent. On the other hand, in the heterogeneity model, as the pH increases the equilibrium concentrations of the acid-base conjugate pair(s) and, thus, the distribution of the charged species that controls the different local environments would change. It has been shown recently that the observed decay rate of the retinal excited state is sensitive to the charge distribution in bR (Song et al., 1993). Thus, one might expect to observe changes in the decay rate of the photoexcited-state of retinal in bR as the pH of the solution is changed.

In this paper, we report the pH dependence of the decay rate of retinal photoexcited state in bR. The results show that a rapid change in the retinal photoexcited-state lifetime occurs in the pH range 9–10, the same pH range in which M_{412} formation rate shows a rapid change (a titration type transition). Our results supports the heterogeneity model in which parallel photocycles each results from retinal having different environment in bR in its ground state contribute to the observed biphasic formation and decay kinetics of the M_{412} intermediate.

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MATERIALS AND METHODS

Bacteriorhodopsin cells were grown from master slants of *Halobacterium halobium* ET1-001 strain kindly supplied to us by Professors R. Bogomolni at UC Santa Cruz. The purple membrane was isolated and purified by a combination of procedures (Oesterhelt and Stoeckenius, 1974; Becher and Cassim, 1975).

Potassium phosphate buffer solutions were used to adjust the pH of the aqueous bR solutions. The final phosphate buffer concentration in bR was about 100 mM. The optical densities of the samples at the absorption maximum ranged from 0.8 to 1.5. Typical transmittance change at 460 nm was between 3 and 7%. All experiments were performed at room temperature (~20°C). All samples were light-adapted under room light for about 30 min before the photoexcited-state lifetime measurements. To minimize contributions from the irreversibly damaged pigment, fresh samples were used and the absorption spectrum of each sample was measured before and after the excited-state lifetime measurements. No significant changes were observed in the absorption spectra of the samples. Thus, the contribution from irreversibly damaged bR pigment can be ruled out.

The laser system and the optical pump-probe setup is similar to that described previously (Song et al., 1993) with minor modifications. Briefly, the laser system consisted of a commercial Coherent Satori dye laser pumped by an Antares mode-locked YAG laser. The output of the dye laser was amplified by a regenerative amplifier (Quantel, RGA 60) in a dye amplifier (Quantel, PTA 60) at 10 Hz. The amplified dye laser beam (at 610 nm with an energy of 800 to 1200 μJ per pulse and a 400 fs pulse width full-width at half-maximum) was split into two beams. One was used as the pump beam and the other was focused into a 5 cm D_2O cell to generate the white light continuum, a portion of which was used to determine the transient absorption. The white light continuum was split into a reference beam and a probe beam. The pump and probe beams were crossed inside the sample cell while the reference beam was sent through the sample but not crossed with the pump beam.

The diameters of the laser beams were ~2 mm. The energy of the pump beam was 20–100 μJ and that of the probe (and the reference) beam was <0.5 μJ. The pump and probe beams were sent through a monochromator to remove the pump beam and to select the detection and reference beams and each was detected by a photodiode. The intensities of the pump, probe, and reference beams were digitized by a computer and the transient absorbance change was recorded. The photoexcitation was initiated with a subpicosecond pulse at λ = 610 nm. The sample cell was either translated vertically at a speed of 2 mm/min to reduce accumulation of long lived photoproducts or the sample was flowed along a 2 mm pathlength flow cell to assure a fresh sample every laser shot. Significant difference in the measured decay rate was observed at pH > 10 when the sample was only translated but not flowed through the sample cell. Data reported here were taken under flow condition.

The photoexcited-state lifetime was monitored either by recording the transient absorption probed at λ = 460 nm or as the ground state bleach recovery at 560 nm as a function of the delay time between the pump pulse and the probe pulse. The transient absorption at 460-nm wavelength is mainly caused by the retinal excited state (Sharkov et al., 1985; Mathies et al., 1988), whereas that at 560 nm is a result of the ground state bleach less the additional absorption of J and K intermediates formed on this time scale. The decay rates and their relative amplitudes are obtained by fitting the signal to a biexponential function convoluted with the instrument response function that was obtained from measuring the ground state bleaching of Rhodamine 640.

RESULTS AND DISCUSSION

Fig. 1 shows the photoexcited-state temporal behavior of the transient absorbance of retinal probed at 460 nm in wt bR at pH 7.8 and 11.3. The open circles are data taken at pH 11.3, and the solid circles are data taken at pH 7.8. The smooth curves running through the circles are results of biexponential fittings convoluted with the instrument response function, which is shown on the left side in Fig. 1. The decay curves are found to fit reasonably well with biexponential functions with small residuals that are caused by the J and K intermediates. Obviously, the photoexcited-state lifetime is longer at pH 11.3 than at pH 7.8. This is reflected from the slower decay as well as the stronger excited-state transient absorption signal at pH 11.3 than at pH 7.8.

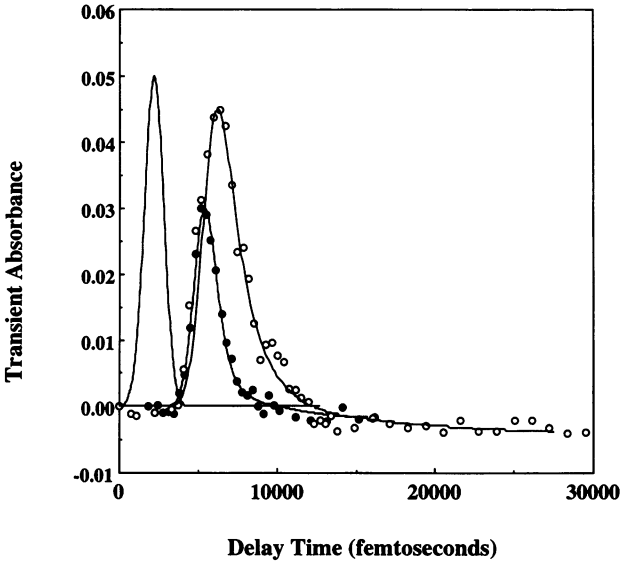

FIGURE 1 Transient absorption of the retinal photoexcited state probed at 460 nm in bacteriorhodopsin at pH 7.8 (●) and 11.3 (○) with solid lines being the results of biexponential fitting. The fitting functions are $\Delta A(t)$ = exp(−t/510) + 0.1*exp(−t/3200) − 0.03 and $\Delta A(t)$ = exp(−t/1300) + 0.1*exp(−t/5000) −0.03 for pH 7.8 and 11.3, respectively (t is in the unit of femtoseconds). These results show that the decay at pH 11.3 is slower than that at pH 7.8, indicating a longer lifetime of the photoexcited state at pH 11.3 than at pH 7.8. The amplitude of the transient absorption signal was also stronger at pH 11.3 than at pH 7.8. The curve on the left is the instrument response function determined from the bleaching of Rhodamine 640.

Fig. 2 shows the pH dependence of the lifetime of the main component of the photoexcited-state of retinal in bR as a function of pH of the bR solution. The solid dots are obtained from transient absorption at 460 nm, and open circles are obtained from the transient absorption probed at 560 nm. Error bars (the uncertainties in the fitting parameters) are also indicated in the figure. The average of the lifetimes obtained at both probe wavelengths is also shown (the curve between the open circles and the solid circles). From Fig. 2, we can see that the lifetime of the photoexcited state of retinal changes rapidly between two pH values in the pH range 9–10. Because our sample was circulated during the experiment to eliminate accumulation of long lived photointermediates or photoproducts, the rapid change of the observed lifetime in this pH range must be associated with at least two different species (whose concentrations are pH-dependent, i.e., involved in acid-base equilibrium) present in the ground state of bR before the photoexcitation. Thus, the dominant absorption at pH < 9 is caused by the retinal in the environment resulting from the low pH species, whereas that at pH > 10 is caused by the retinal whose environment is

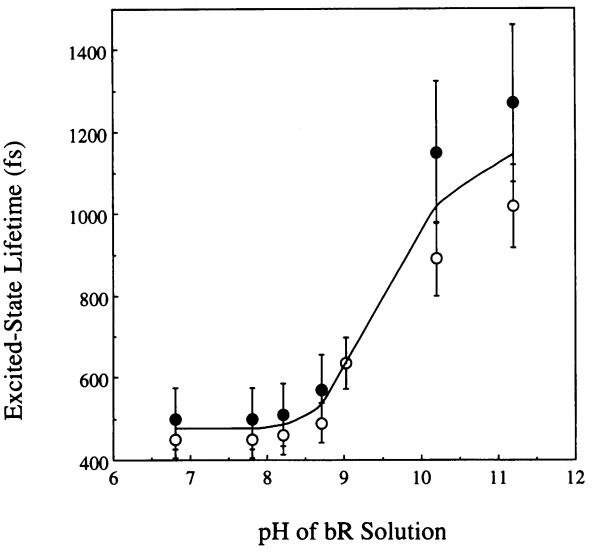

FIGURE 2 Dependence of the main component of retinal excited-state lifetime on the pH of the bR solution. Solid circles were obtained from the transient absorption probed at 460 nm, and the open circles were obtained from the transient absorption probed at 560 nm. A rapid change occurs at pH between 9 and 10, similar to that observed for the M_{412} formation kinetics.

affected by the high pH species of the acid-base equilibrium of the amino acid(s) controlling the retinal environment.

From Fig. 2, the change in the observed lifetime occurs at pH ~ 9.5. This titration-type change in the excited-state lifetime of bR has a similar pKa value as that responsible for the formation of the alkaline bR (bR_a) reported previously (Balashov et al., 1991). The question arises as to which amino acid residue with a pK_a of ~9.5 is responsible for controlling the retinal excited state lifetime and what is the mechanism involved? The pK_a's of the side chain of tyrosine, aspartic acid, and arginine in aqueous solution are 10.1, 3.9, and 12.5, respectively (Voet et al., 1990). These values can be changed dramatically in the protein depending on the dielectric constant and the charge distribution around the amino acids. The pKa values of the aspartic acids in bR vary from less than 2 for Asp-85 (Jonas et al., 1991) to greater than 7 for Asp-96 and Asp-115 (Braiman et al., 1988). These numbers are dramatically different from the pK_a value in aqueous solution. Thus, the amino acid residue(s) responsible for the observed lifetime change is not easy to assign. The most favorable candidate is a tyrosine residue (Bolgomolni et al., 1978; Hess et al., 1979; Rosenbach et al., 1982; Hanamoto et al., 1984; Ovchinnikov et al., 1986; Balashov et al., 1991). However, recent studies are controversial regarding the protonation/deprotonation status of Tyr residues at high pH, although its pK_a in aqueous solution is 10.1. According to a recent resonance Raman study, there was no tyrosinate observed at pH as high as 11 (Ames et al., 1990). This observation was consistent with a solid state NMR measurement that the pK_a of tyrosine in bR is about 13 (Herzfeld et al., 1990). On the contrary, in a different study, it was concluded that Tyr-185 or Tyr-57 and possibly a few other tyrosines (Balashov et al., 1991) are deprotonated at pH 11. Our results suggest that one or more amino acid residue(s) changes from one form to the other at a solution pH of ~9.5.

Recently, the quantum yield of the retinal photoisomerization has been determined (Logunov et al., 1994) in the 6.8–11.3 pH range. It was also concluded that the observed change in the rate of the excited state decay paralleled that for the retinal photoisomerization process in this pH range. To understand the effect of heterogeneity on the photoisomerization dynamics, we must discuss the possible mechanisms responsible for the rapid photoisomerization of retinal in bR. The photoisomerization of protonated Schiff base of all-*trans* retinal in methanol solution occurs in ~5 ps (Kandori et al., 1993), and the main photoproduct was predominantly the 11-*cis* isomer. However, the photoisomerization of the all-*trans* retinal in bR is catalyzed by a factor of 10 and becomes highly specific to give the 13-*cis* isomer in bR (Mathies et al., 1988; van den Berg et al., 1990; Song et al., 1993). This could result from at least two factors. The first is steric in nature. The absence of vibronic structure in its absorption spectrum suggests (Schreckenbach et al., 1978; Fahmy et al., 1990) that the retinal in bR is not quite planar. This must result from steric forces between the methyl groups along the retinal chain and the amino acid residues of the protein within the retinal cavity. Such steric repulsive interactions must then be relieved upon photoisomerization. In a recent study, the effect of amino acid replacement of charged and hydrogen-bonding residues within the retinal cavity on the photoisomerization dynamics was reported (Song et al., 1993). Negatively charged Asp-85 and Asp-212 are found to catalyze the photoisomerization process. This might result from changing the extent of intermolecular steric effect by slightly changing the conformation of the protein molecule around the retinal within the cavity but should undoubtedly change the electronic charge distribution of the retinal in its excited state, e.g., by changing the C_{13}-C_{14} bond order. The latter was proposed (Song et al., 1993) to result from charge stabilization of the ionic form having a positive charge on the C_{13} of retinal in the retinal excited state by the aspartate negative charges of Asp-85 and Asp-212, which are only a few Å away.

A typical acid-base equilibrium involves two species, one charged and the other one neutral. The electrostatic environment of the bR molecules whose retinals are affected by the charged species is expected to be different from that with the retinals affected by the neutral species. Changing one form into the other, as the pH changes, could lead to changes in either the steric and/or the electronic contribution to the protein catalysis of the retinal photoexcited-state decay in bR. If the amino acid that controls the environment is near the retinal, a direct coupling between its charged form could increase the bond order of the C_{13}-C_{14} bond of retinal either by destabilizing the positive charge on C_{13} if Arg-type residue is involved in the equilibrium or by stabilizing the positive charge on the other odd carbon atoms on the retinal chain if the acid-base equilibrium involves Asp or Tyr residues (see the catalysis mechanism given in Song et al., 1993). If the

environmentally controlling amino acid is far from the retinal, it could indirectly change the protein conformation around the retinal so that the steric and/or the electronic factors change (by changing the orientation of the O^- of Asp-85 or Asp-212 with respect to the C_{13^+} of the retinal in the photoexcited state). Thus, direct coupling of the environmentally controlling amino acid residues could be electrostatic either with the C_{13^+} of retinal in the excited state or with either aspartate Asp-85 or Asp-212. Indirect coupling could change the conformation of the retinal pocket, thus changing either the distance between C_{13} of retinal and Asp-85 or Asp-212, the water structure, and thus modifying the dielectric constants within the cavity, or else it changing the steric factors resulting from the repulsive interaction between the methyl groups on the retinal and the nearby amino acid group within the protein cavity.

Different proposals can be discussed regarding which amino acid residues are responsible for the observed heterogeneity. In the original proposal (Hanamoto et al., 1984), Tyr was proposed because the pKa value of the responsible amino acid(s) was found to be near 9.5. Later, Tyr-57 (Balashov et al., 1991), Tyr-185 (Ovchinnikov et al., 1986; Rothschild et al., 1989; Balashov et al., 1991), and possibly Tyr-64, Tyr-79, Tyr-131, and Tyr-133 (Balashov et al., 1991), as well as Asp-96 (Fukuda et al., 1992) were proposed to be the deprotonated amino acid(s) in this high pH range. More recently, it was found that the rate of the radiationless processes (i.e., the internal conversion process) in bR also decreases in the same pH range (Logunov et al., 1994). A systematic study of the pH dependence of the retinal excited-state decay as well as the rate of radiationless processes of retinal in bR in different mutants might assist in identifying the amino acid(s) responsible for the observed heterogeneity of the bR photocycle kinetics.

CONCLUSION

By examining the pH dependence of the decay rate of the photoexcited state of retinal in bR, it is concluded that acid-base equilibrium induces heterogeneity in the retinal environment of bR ground state. This gives rise to at least two types of pH-sensitive environments of the retinal in bR. Upon photoexcitation, at least two parallel photocycles are initiated in bR whose retinal photoexcited states have different decay rates. At low pH, the observed excited-state lifetime is determined by the component whose environment is determined by the low pH-dominant species in the acid-base equilibrium of the amino acid residue that controls the charge distribution of the retinal environments. At high pH, the observed lifetime is that for the retinal whose environment is determined by the high pH species of an acid-base equilibrium with a pKa value of ~9.5. Our results also suggest that this kind of heterogeneity observed in the primary step of the bR photocycle must contribute to the previously observed apparent pH dependence of the M_{412} kinetics.

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