Interaction between Asp-85 and the Proton-Releasing Group in Bacteriorhodopsin. A Study of an O-like Photocycle Intermediate†

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ABSTRACT: Upon light adaptation by continuous (or pulsed) illumination, the artificial bacteriorhodopsin (bR) pigments, I and II, derived from synthetic 14F retinal and a short polyenal, respectively produce a long-lived red-shifted species denoted O_l. An analogous phenomenon was observed by Sonar, S., et al. [(1993) *Biochemistry 32,* 2263-2271], in the case of the Y185F mutant (pigment III). The nature of these O_l species was investigated by studying a series of effects, primarily their red light photoreversibility, the associated proton uptake and release processes, and the effects of pH on their relative amounts, which are interpreted in terms of pH-dependent acid−base equilibria. Experiments were also carried out with pigments I and II derived from the mutants D96A, E204Q, R82Q, and D85N. The O_l species of pigments I and II (and possibly also that of pigment III) are identified as an unusually long-lived (*all-trans*) intermediate of the photocycle of their 13-*cis* isomer. It is concluded that in O_l, Asp-85 is protonated, a process associated with proton uptake from the extracellular side. Subsequent proton release (to the same side of the membrane) occurs from Glu-204 (or from a group closely interacting with it) prior to the decay of O_l. At high pH (>9), O_l reversibly converts to a purple form, due to deprotonation of Asp-85, while at still higher pH (>11), a blue-shifted species characterized by a deprotonated Schiff base is generated. These transitions constitute the first demonstration of the titration of a photocycle intermediate of a retinal protein. The respective pK_a values are determined and discussed in relation to those pertaining to the unphotolyzed (dark-adapted) pigments. It appears that the pK_a values are controlled by a hydrogen bond network involving water molecules, which binds the protonated Schiff base with Asp-85 and Glu-204. The disruption of this network in pigments I-III may also be responsible for the long lifetime of the O_l species, due to the inhibition of thermal *trans*−13-*cis* isomerization. The results are relevant to the molecular mechanism of the photocycles of both 13-*cis*- and *all-trans*-bR, primarily to the nature and to the deprotonation mechanism of the proton-releasing group.

Bacteriorhodopsin (bR),[1] the retinal protein of *Halobacterium salinarium*, functions as a light-driven proton pump leading to ATP synthesis [for a recent comprehensive series of review articles on bR, see Ottolenghi and Sheves (1995)]. The proton-pumping activity is associated with the *all-trans* configuration of the retinylidene chromophore, which is attached to the protein via a protonated Schiff base linkage (Lewis et al., 1974) with Lys-216. The photocycle of the *all-trans* chromophore is associated with the following simplified sequence of reactions:

$$bR_{570} \xrightarrow{h\nu} K_{590} \rightarrow L_{550} \rightleftharpoons M_{412} \rightleftharpoons N_{550} \rightleftharpoons O_{640} \rightarrow bR_{570}$$

in which subscripts refer to the maximum absorption of the respective intermediate. After chromophore photoisomerization to the 13-*cis* configuration in K_{590}, the Schiff base transfers a proton to Asp-85 in the $L_{550} \rightarrow M_{412}$ stage. This process is accompanied by proton release to the extracellular side by a group denoted as XH. XH is most probably associated with a complex including Glu-204, Arg-82, Tyr-57, other protein residues, and several structured water molecules. It was recently suggested that XH should be identified as Glu-204 (Brown et al., 1995). The Schiff base reprotonates from the cytoplasmic side via Asp-96 during the $M_{412} \rightarrow N_{550}$ transition, while the original *all-trans* configuration is regained in O_{640}. The photocycle is completed by direct reprotonation of X^- by Asp-85.

The photocycle of 13-*cis*-bR (Kalisky et al., 1977; Sperling et al., 1977; Hofrichter et al., 1989; Steinberg et al., 1994) exhibits primary events analogous to those of *all-trans*-bR, but at neutral pH, it does not involve deprotonation of the Schiff base and does not lead to proton translocation. The related events are described by the photocycle

$$bR\ (13\text{-}cis) \xrightarrow{h\nu} K^c \rightarrow L^c_{610} \rightarrow bR\ (13\text{-}cis)$$

in which K^c is analogous to K_{590} and L^c_{610} is a photorevers-

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[1] Abbreviations: BM, blue membrane; bR, bacteriorhodopsin; DA, dark-adapted; D96A, bacteriorhodopsin mutant in which Asp-96 is replaced by alanine; E204Q, bacteriorhodopsin mutant in which Glu-204 is replaced by glutamine; LA, light-adapted; PSB, retinal protonated Schiff base; PM, purple membrane; R82Q, bacteriorhodopsin mutant in which Arg-82 is replaced by glutamine; WT bR, wild-type bacteriorhodopsin.

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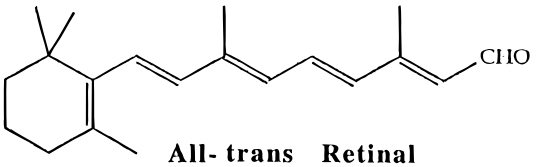
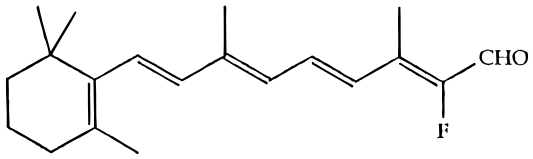
Chart 1



All- trans Retinal



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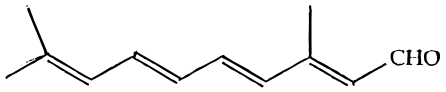

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ible red-shifted interemediate. The reasons for the lack of M formation and proton pumping capability in the 13-*cis* photocycle at neutral pH and their reappearance at high pH and in some artificial bR molecules have been recently discussed (Drachev et al., 1993; Steinberg et al., 1994). It is evident that clarification of these phenomena as well as establishing the relationship between L^c_{610} and the late photocycle intermediates (primarily O_{640}) in the photocycle of bR_{570} (*trans*) are crucial in understanding the molecular mechanism of the photocycle and of the related proton pump.

Recently, Rothschild and co-workers (Sonar et al., 1993; Rath et al., 1993; He et al., 1993) have approached the structure and function of the key intermediate (O_{640}) by studying the properties of the Tyr-185 → Phe mutant (Y185F) of bR_{570}. The light-adapted form of this mutant revealed an equilibrium between its purple form and an O-like species (which in this paper will be denoted as O^{III}_{608}; see below). It was suggested that O^{III}_{608} is analogous to the O_{640} photointermediate of the native system, with the distinction that it is already present in the dark in thermal equilibrium with the purple form (Ahl et al., 1988; Sonar et al., 1993).

In the present work, we have reexamined the above issues after having recently observed that properties analogous to those of Y185F are also observed in the wild-type (WT) pigment upon replacement of the native *all-trans* chromophore with the two synthetic chromophores 1 and 2 (Chart 1). We have analyzed the spectroscopic properties and pH effects pertaining to these two artificial pigments (denoted as bR^I and bR^{II}, respectively) as well as those of the Y185F mutant (which we denote as bR^{III}). We show that upon light adaptation all three pigments exhibit analogous O-like intermediates (O^I_{640}, O^{II}_{510}, and O^{III}_{608}), which we generally denote as O_l. The work was carried out in both WT bR and in several bR mutants involving protein residues (Asp-96, Arg-82, and Glu-204), which play key roles in the light-induced function of bR. In the case of pigments I and II (and probably also III), the data suggest an alternative identification of the O_l species in terms of the photocycle of the 13-*cis* isomer of the parent pigment. O_l was found to exhibit pH-dependent equilibria with a purple and with a yellow form of the light-adapted pigments, which were interpreted in terms of titration of Asp-85 and the protonated Schiff base, respectively. Such observations represent the first successful titration of a photocycle intermediate of a rhodopsin pigment. The results bear directly on the relationships between the 13-*cis*- and *all-trans*-bR photocycles and, primarily, on the mechanisms of Asp-85 protonation and of proton release by XH in the proton pump of bacteriorhodopsin.

MATERIALS AND METHODS

Artificial and Mutant bRs. The synthetic chromophores 14F retinal (1) and the short polyenal (2) were synthesized according to previously described methods (Maradin-Szewezkowska et al., 1984; Asato et al., 1978). bR mutants Y185F, D96A, E204Q, and R82Q were obtained as a generous gift from Profs. R. Needleman and J. Lanyi. The artificial pigments were prepared by reconstituting the apomembrane with the synthetic retinal. Preparation of apomembrane and pigment reconstitution were carried out using previously described methods (Tokunaga & Ebrey, 1978).

Pulsed Laser Photolysis. A dark-adapted sample of pigment I was exposed to 532 nm, 9 ns pulses from a Nd-YAG laser. To avoid irradiation of the photoproducts, the pulses were delayed by 4 min. Laser-induced absorbance changes were recorded using a continuous 75 W Xe lamp, a photomultiplier, and a TDS-520 Tektronix digitizer. Data were averaged and analyzed using a personal computer. To minimize light adaptation by the monitoring light, interference and neutral density filters were placed between the Xe lamp and the sample.

Absorption Spectroscopy of the Pigments. Light adaptation was carried out using a light source (halogen lamp, 150 W) equipped with an optical fiber light guide (Schott KL 1500, Germany). Exposure of the dark-adapted forms of the pigments (at a concentration of 10^{-5} M) to 10−20 s illumination was sufficient for generating the red-shifted photoproducts. bR^{III} (Y185F) was irradiated using a $\lambda >$ 520 nm cutoff filter. The artificial pigment bR^I_{588} derived from 14F retinal was irradiated using a $\lambda >$ 550 nm cutoff filter, while bR^{II}_{450} was irradiated through a $\lambda =$ 440 nm interference filter.

Determination of pK_a values for the purple to blue membrane transition was carried out by titration of dark-adapted suspensions in aqueous solution without salt or with 10 mM/0.1 M NaCl, as indicated, the pH being adjusted with HCl and NaOH. The titrations were carried out as fast as possible to avoid aggregation.

Measurements of Absorption Changes of pH Indicator Dyes. Since kinetic measurements of light-induced pH changes were carried out over a relatively large pH range (pH 5.5−pH 9.0), the following indicators with pK_a values covering this range were used: pyranine, chlorophenol red, bromothymol blue, and thymol blue. Bromothymol blue was preferred over pyranine at neutral pH in the measurements involving bR^{II}_{450}, since the absorption maximum of this pigment is close to the absorption maximum of the basic form of pyranine (450 nm) whereas the basic form of bromothymol blue absorbs at longer wavelengths (574 nm). For the same reason, pyranine was preferred over bromothymol blue at neutral pH in measurements involving the

Table 1: Absorption Maxima (Nanometers) of Pigments I−III and Mutants E204Q, D96A, R82Q, and D85N at Neutral pH[a]

pigment	DA	LA	DS
bR (wild type)	560	568	588/504
bR^{I} (14F retinal)	588	610	640/534
bR^{III} (Y185F)	548	566	608/506
bR^{II} (polyene 2)	450	480	510/426
$bR^{I}_{(D96A)}$	588	610	638/544
$bR^{I}_{(E204Q)}$	586	610	670/570
$bR^{I}_{(R82Q)}$	590	610	664/556
$bR^{II}_{(D96A)}$	450	480	508/420
$bR^{II}_{(E204Q)}$	446	450	500/410
$bR^{II}_{(R82Q)}$	450	462	510/430
$bR^{II}_{(D85N)}$	480	480	−

[a] DS refers to the absorption maxima in the difference spectra of the dark-adapted (DA) sample subtracted from the spectra of the light-adapted (LA) sample (LA − DA).

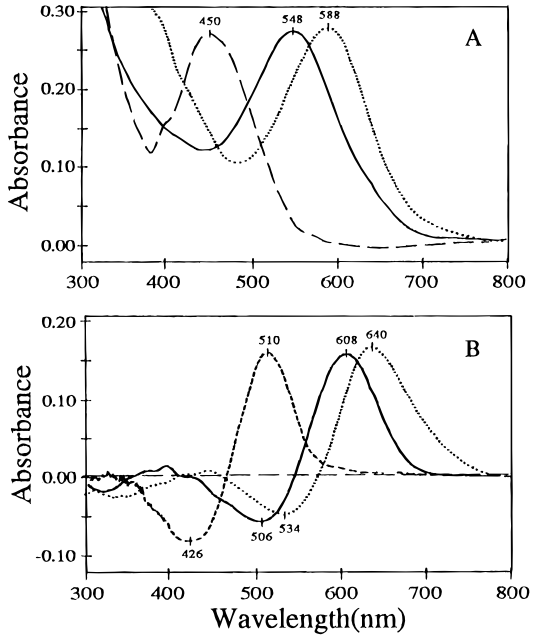


FIGURE 1: Absorption spectra of (···) bR^{I}_{588}, (− −) bR^{II}_{450}, and (—) bR^{III}_{550}: (A) dark-adapted forms and (B) LA − DA difference spectra.

other pigments, which absorb at longer wavelengths (∼560 nm). The absorption changes of the dye indicators were monitored in pigment solutions containing 0.1 M NaCl. The pH was adjusted by addition of HCl and NaOH. Control experiments aiming at the cancellation of the dye effect were carried out by adding 10 mM phosphate buffer to this solution.

RESULTS

UV-Visible Absorption Spectra of the Red-Shifted O_I Species. It was shown previously that, in contrast to wild-type bR, in which light adaptation causes a relatively small spectral shift (from 560 to 568 nm), steady state illumination of the artificial pigments bR^{I}_{588} (I) and bR^{II}_{450} (II) and of the mutant Y185F (bR^{III}_{550}) formed photoproducts which are markedly red-shifted with respect to their parent pigments (see Table 1 and Figure 1). As mentioned above and for reasons that will be discussed below, these red-shifted species are denoted as O^{I}_{640}, O^{II}_{510}, and O^{III}_{608}, respectively [subscripts indicate the absorption maxima of the red-shifted band in the light-adapted (LA) minus dark-adapted (DA) difference spectra]. In keeping with the previous findings of Sonar et al. (1993), we find that the thermal decay of O^{III}_{608} back to the original purple pigment (bR^{III}_{550}) is characterized by a single exponential with a half-life ($\tau_{1/2}$) of 13 h at neutral pH. O^{II}_{510} and O^{I}_{640} are less thermally stable, being characterized at neutral pH by $\tau_{1/2}$ values of 300 s and 60 s, respectively. All three O_I species have previously been found to be characterized by an *all-trans* configuration (Tierno et al., 1990; Steinberg et al., 1991; He et al., 1993). It is important to note that O^{I}_{640} and O^{II}_{510} are photoreversible. Thus, upon illumination with red light, a large fraction (80% in O^{I}_{640} and O^{II}_{510}) of the red-shifted species reconverts to the parent pigment. No detectable amount of O^{III}_{608} was found to revert to its parent pigment following illumination.

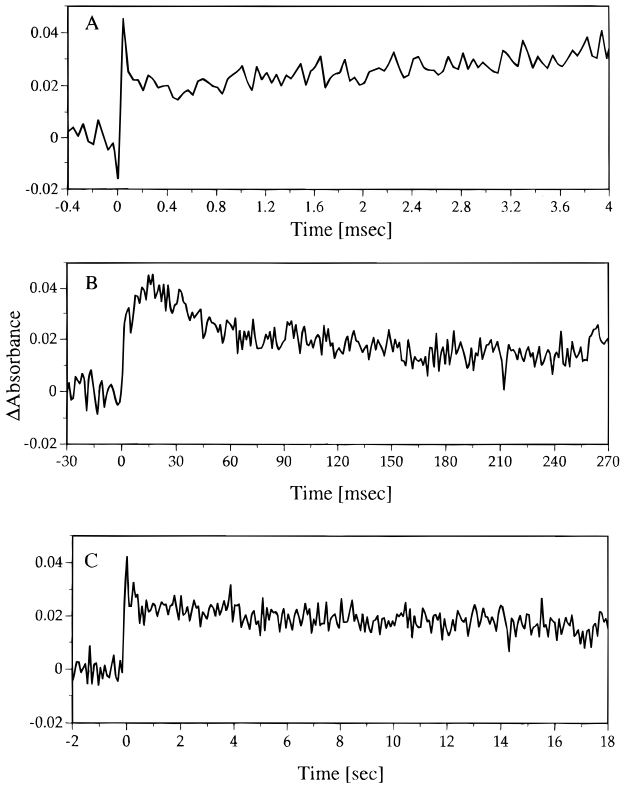


FIGURE 2: Pulsed laser photolysis patterns of bR^{I}_{588}. Trace A at 640 nm shows an initial decay component ($\tau_{1/2} \sim 50\ \mu s$) attributed to the decay of the K intermediate and a slow growing in due to the O intermediate of the *trans* photocycle. Trace B at 640 nm represents the growing in ($\tau_{1/2} \sim 7$ ms) and decay ($\tau_{1/2} \sim 30$ ms) of the O intermediate. Trace C at 640 nm represents the slow (∼60 s) decay of O_I intermediate.

Flash Photolysis Experiments of bR^{I}_{588}. With the purpose of the investigation of the photocycle of the dark-adapted form of pigment bR^{I}_{588}, the latter was exposed to laser photolysis experiments under conditions of negligible accumulation of O^{I}_{640}. This was achieved by minimizing the intensity of the probe beam with appropriate interference and neutral density filters and by using a relatively long (∼4 min) delay between consecutive exciting laser pulses. (Due to the relatively long lifetimes of O^{II}_{510} and O^{III}_{608}, similar experiments were not carried out with bR^{II}_{450} and bR^{III}_{550}.) As shown in Figure 2, the major photolysis features of bR^{I}_{588} consist of a red-shifted K intermediate, decaying to an (blue-shifted) M intermediate in ca. 0.1 ms (Figure 2A) and a

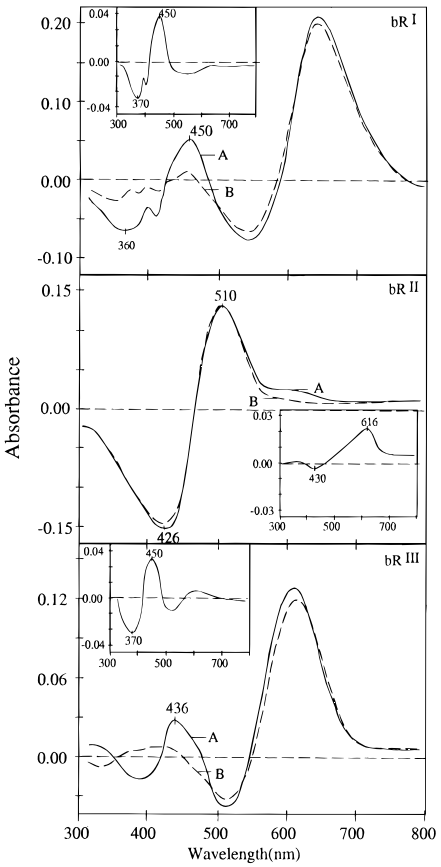


FIGURE 3: Absorption difference spectra (LA − DA) of bR^{I}, bR^{II}, and bR^{III} at pH 7.2 (A) with dye in the bulk and (B) with dye and phosphate buffer in the bulk. (Inset) Absorption difference spectra of A − B. Pyranine was used in cases of bR^{I} and bR^{III}. Bromothymol blue was used with bR^{II}.

growing- in of a red-shifted O species (Figure 2A,B) in ca. 15 ms, as previously reported by Tierno et al. (1990). However, we also observe (Figure 2C), superimposed on these features, which are characteristic of the photocycle of *all-trans*-bR, a long-lived red-shifted species whose (∼60 s) lifetime matches the decay of O^{I}_{640} as measured in our steady state illumination experiments. Note that the relative amount of contribution of the O^{I}_{640} intermediate (above 50%) is in keeping with the relative amount of 13-*cis* pigment in the dark-adapted form of the Y185F pigment (He et al., 1993).

Proton Uptake and Release. Monitoring the transient absorption changes of pH indicator dyes which are present in the aqueous bulk during irradiation of the pigments shows that formation of the red-shifted photoproduct is associated with a proton uptake process. Figure 3 represents the LA minus DA difference spectra of bR^{I}, bR^{II}, and bR^{III} at pH 7.2 (unbuffered) in the presence of a pyranine indicator in the aqueous bulk (for bR^{I} and bR^{III}) and of bromothymol blue (for bR^{II}). It is shown that the appearance of O^{I}_{640}, O^{II}_{510}, and O^{III}_{608} is accompanied by the formation of the basic form of the indicator (λ_{max} = 450 nm for pyranine and 616 nm for bromothymol blue) and by bleaching of the respective acidic form. This light-induced dye effect is canceled when 10 mM phosphate buffer (pH 7.2) is present in the bulk solution (Figure 3, trace B). An important finding is that, while the half-life values of O^{I}_{640} and O^{II}_{510} are 1 and 5 min, respectively, those corresponding to restoration of the original pH are 8 s and ca. 2 min, respectively (see Figure 4). We

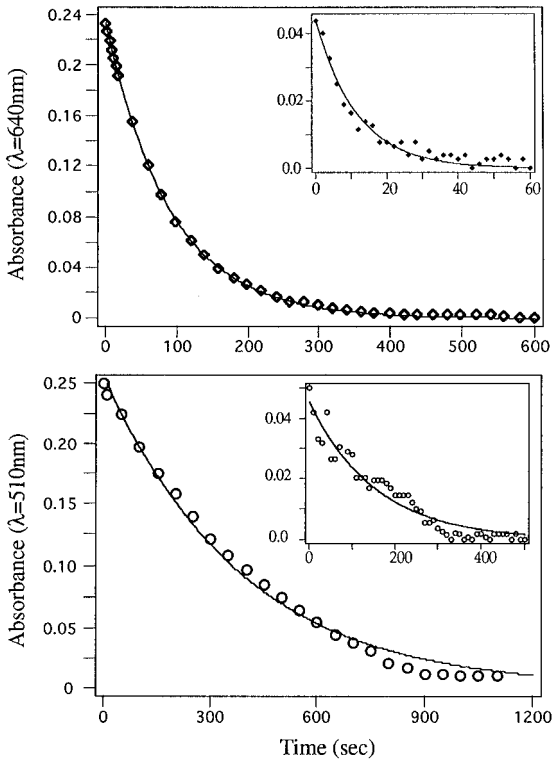


FIGURE 4: Thermal decays of O^{I}_{640} (upper) and O^{II}_{510} (lower) at pH 7.2. (Insets) Decay of the pyranine basic form (λ_{max} = 450 nm) which is present in the aqueous bulk during irradiation of the pigments.

note that the lifetime of O^{III}_{608} is extremely long (ca. 13 h), making it difficult to exactly monitor the relatively weak changes experienced by the pyranine dye over this period. A rough estimate is that in this case the original pH is restored after ca. 10 min. These results imply that in all cases the proton release process, which follows the uptake event, substantially precedes the decay of the red-shifted photoproduct. Similar uptake and release processes were also observed at pH 5. Here too, the proton release process preceded the thermal decay of O_l, exhibiting kinetics similar to those observed at pH 7.

pH Effects on the Yields of O^{I}_{640}, O^{II}_{510}, and O^{III}_{608}. It was shown previously that the yield of O^{III}_{608} decreases at high pH, exhibiting titration-like behavior, with a pK_a of ∼9.0. The effect was attributed to an equilibrium between the *all-trans* form of bR^{III}_{550} and its O photocycle intermediate (Sonar et al., 1993). Similarly, we have observed that upon illumination the yields of O^{I}_{640} and O^{II}_{510}, which are relatively high (40%) at neutral pH, decrease markedly at high pH (see Figure 5). In complete analogy to the observations of Sonar et al. (1993) in the case of pigment III, we observed that an immediate pH reduction in the dark of a light-adapted sample of both I and II (from high to neutral pH) generated the red-shifted species in an amount similar to that obtained after illumination at pH 7. Moreover, immediate pH elevation following irradiation at pH 7 leads to similar depletion of the red-shifted species to a light-adapted sample irradiated at high pH. This reversibility of the O_l titration is consistent with the existence of a pH-dependent equilibrium between the red-shifted O_l species and a purple one absorbing in a manner similar to that of the parent pigment. As shown in Figure 6 A,B and Table 2, pK_a values of 8.7 and 9.05 were determined for O^{I}_{640} and O^{II}_{510} (10 mM NaCl),

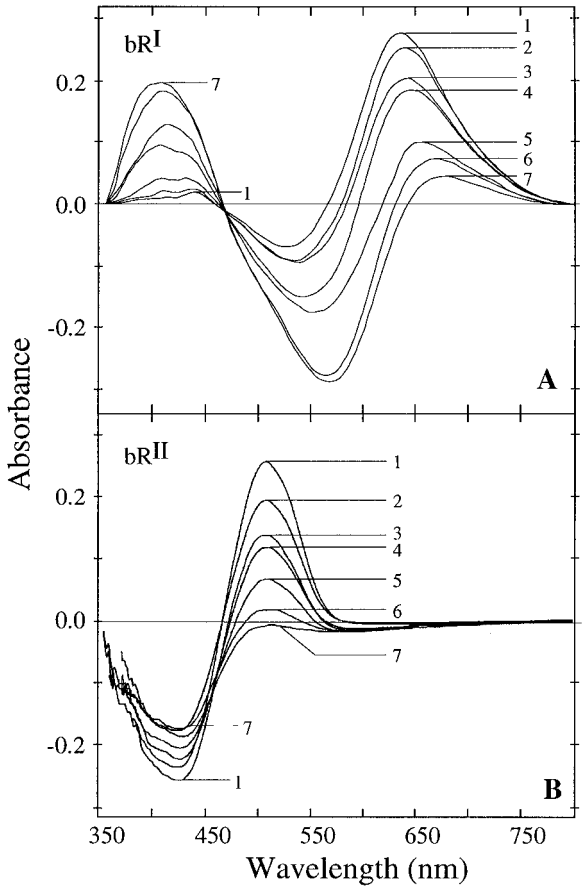


FIGURE 5: Absorption difference spectra (LA − DA) of bR^I and bR^{II} in 10 mM NaCl at various pH values. (A) bR^I_{588} at pH values of 7.3 (1), 7.6 (2), 8.1 (3), 8.6 (4), 9.0 (5), 9.3 (6), and 9.6 (7). (B) bR^{II}_{450} at pH values of 8.4 (1), 8.6 (2), 9.0 (3), 9.3 (4), 9.6 (5),10.0 (6), and 10.5 (7).

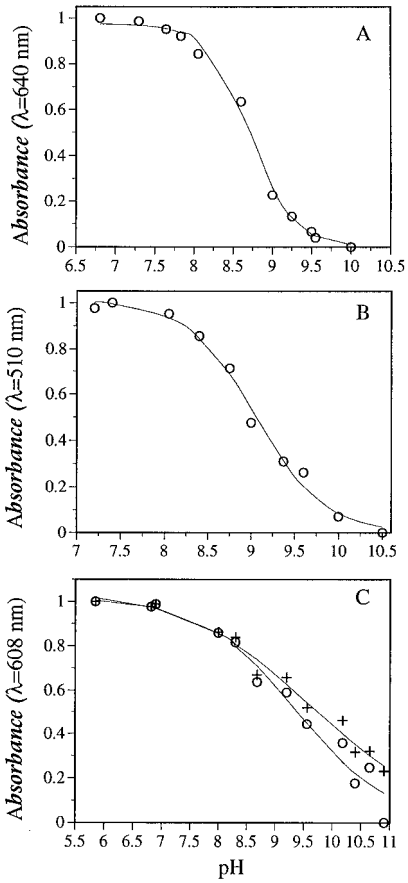


FIGURE 6: Titration curves of O^I_{640} (A), O^{II}_{510} (B), and O^{III}_{608} (C) in 10 mM NaCl. In panel C, values are given before (○) and after (+) the proton release process. (○) Points were taken immediately after illumination; (+) points were taken (2 min) after illumination. The solid line represents the best-fit titration with a pK_a of 8.70 ± 0.03 (n = 1.6 ± 0.2) (A) and a pK_a of 9.05 ± 0.04 (n = 1.1 ± 0.10) (B). (C) pK_a = 9.40 ± 0.1 (n = 0.6 ± 0.1) (○); pK_a = 9.6 ± 0.1 [n = 0.6 ± 0.1 (+)].

respectively, by measuring the relative amounts of O_l generated from the parent pigments at different pH values.

As reported above, the light-induced proton release process which follows the uptake reaction is considerably faster than the thermal decay of O_l. The phenomenon is especially pronounced in the case of bR^{III} (Y185F). We have been able to determine the pK_a of the above transformation, before and after the proton release process, by detecting the yield of O^{III}_{608} at various time intervals after illumination. It was shown that an additional fraction of O^{III}_{608} is generated during the first several minutes in the dark at pH ≥8. This indicates that the pK_a value of the red-shifted intermediate is slightly increased during the proton release process. Figure 6C shows an increase from a pK_a of 9.4 to 9.6 following the proton release process.

Titration of the Protonated Schiff Base of the O_l Species. As described above, irradiation of pigment II at relatively high pH (between 7 and 10) reduces the yield of O^{II}_{510} due to its titration to a species absorbing in a manner similar to that of the parent pigment. However, as shown in trace A of Figure 7, irradiation of the DA form at still higher pH values (10−12) reveals the formation of a blue-shifted species absorbing at 350 nm and parallel bleaching of the parent pigment absorption at 450 nm. As shown in Figure 8A, this titration is characterized by a pK_a of 11.6 ± 0.2. Both the high pK_a value and the 350 nm absorption, which is characteristic of nonprotonated Schiff bases, are consistent with the titration of the Schiff base of O^{II}_{510}. This is further confirmed by the observation that, in the dark, the 350 nm species thermally regenerates the DA form with a $\tau_{1/2}$ of approximately 5 min, which fairly corresponds to the decay time of O^{II}_{510}. Moreover, illumination at neutral pH followed by elevation of the pH to 12 led to the formation of the same blue-shifted intermediate, in keeping with the conclusion that the 350 nm species originates from a higher

Table 2: Apparent pK_a Values of Equilibria Associated with Photoinduced Species of bR^I, bR^{II}, and bR^{III}

pK_a values for the photo-induced O_l species attributed to the Asp-85 titration			pK_a of the photo-induced 350 nm M_l species attributed to the Schiff base titration	
bR^I	bR^{II}	bR^{III}	bR^I	bR^{II}
8.7[b]	9.05[b]	9.0[a]	8.9[b]	11.6[b]
		9.4[c]		
		9.6[d]		
$bR^I_{(D96A)}$	$bR^{II}_{(D96A)}$			
8.7[b]	9.05[b]			
$bR^I_{(R82Q)}$	$bR^{II}_{(R82Q)}$			
8.7[b]	9.05[b]			
$bR^I_{(E204Q)}$	$bR^{II}_{(E204Q)}$		$bR^I_{(E204Q)}$	$bR^{II}_{(E204Q)}$
6.9[b]	7.0[b]		7.0[b]	9.0[b]

[a] Sonar et al. (1993). [b] 10 mM NaCl. [c] 10 mM NaCl, immediately after illumination (prior to proton release). [d] 10 mM NaCl, 2 min after illumination (after proton release).

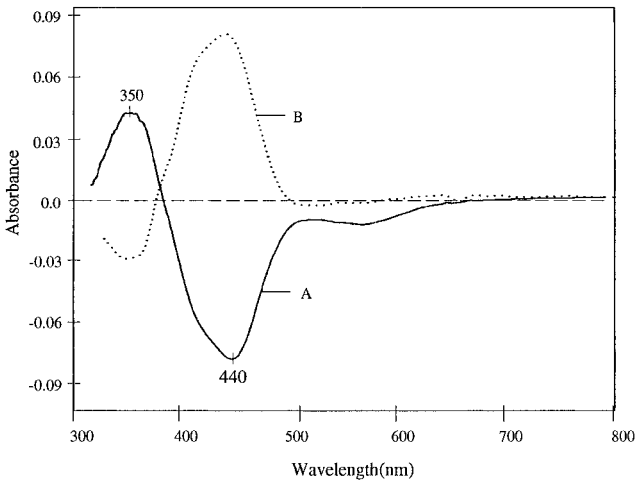


FIGURE 7: Absorption difference spectra of bR^{II} at pH 12. (A) LA − DA (illumination with interference filter, $\lambda = 440$ nm). (B) Difference spectrum obtained by subtracting the original LA spectrum from the one obtained after subsequent irradiation with red light ($\lambda > 550$ nm).

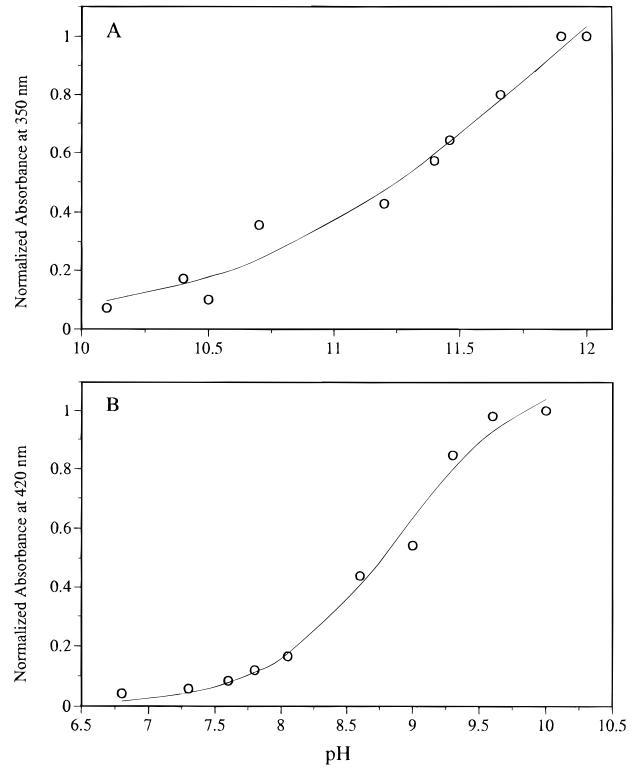


FIGURE 8: Titration curves of the protonated Schiff base of O^{II}_{510} (A) and O^{I}_{640} (B) in 10 mM NaCl. The solid line represents the best titration fit with a pK_a of 11.6 ± 0.2 ($n = 0.9 \pm 0.1$) (A) and a pK_a of 8.9 ± 0.1 ($n = 0.9 \pm 0.1$) (B).

pH titration of the same system. The reverse titration (a LA sample at pH 12 was acidified in the dark to pH 7) led to the recovery of the red-shifted species at the expense of the 350 nm species. Thus, the process of illumination at very high pH is composed of two stages: generation of O^{II}_{510} from the parent pigment and its titration to the blue-shifted species. The existence of an equilibrium between the 350 nm species and O^{II}_{510} is further supported by the observation that irradiation of the LA form at pH 12 with red light ($\lambda >$ 550 nm, which is absorbed by the small amount of O^{II}_{510} still present, but not by the predominant 350 nm species) leads to the depletion of the 350 nm band (Figure 7, trace B).

Phenomena which are basically analoguous to the above were also observed with O^{I}_{640}. As previously presented in Figure 5, the LA minus DA difference spectra for bR^{I}, recorded over the range of pH 7.0−10.0, show that at high pH the relative amount of O^{I}_{640} decreases. This process is compensated for by the generation of a blue-shifted species absorbing at 420 nm. Immediate pH elevation of a LA sample from 7 to 10 indicated the formation of the 420 nm species from O^{I}_{640}. A reverse process took place following pH reduction from 10 to 7. These experiments demonstrate that in a manner analogous to O^{II}_{510}, O^{I}_{640} is titrated to a blue-shifted (420 nm) species, characterized by a nonprotonated Schiff base. However, as shown in Figure 8B, the titration of O^{I}_{640} exhibits a pK_a of 8.9 ± 0.1, which is lower relative to that of O^{II}_{510}. This is consistent with the fact that the pK_a of the protonated Schiff base in the parent pigment is already reduced, from 13.3 in WT bR to 11.5 (Steinberg et al., 1994; Govindjee et al., 1994), due to the electron-withdrawing fluorine substitution at position 14 which destabilizes the positive charge of the protonated Schiff base form (Sheves et al., 1986). In O^{I}_{640}, the pK_a of the protonated Schiff base is further decreased to 8.9. In other words, the same pK_a difference of about 2 units is observed in both bR and O_l systems. Thus, whereas in pigment II two distinct pK_a's were found at pH 9.05 and 11.6 for generation of O^{II}_{510} and titration of its protonated Schiff base, respectively, in pigment I, both processes have similar pK_a values (8.7 and 8.9). We finally note that we failed to obtain the pK_a value of the protonated Schiff base of O^{III}_{680}, since at high pH the pigment experienced decomposition.

Artificial Pigments of Mutants D96A, E204Q, R82Q, and D85N. To investigate the possible involvement of key protein residues in the formation of the O-like species, as well as in the related proton uptake and release activity, we studied the artificial pigments of mutants D96A, E204Q, R82Q, and D85N in which the native chromophore was replaced by the synthetic 14F retinal **1** or by polyene **2**. The absorption maxima of these artificial mutants were similar to those of the respective artificial wild-type pigments (Table 1), except for $bR^{II}_{(D85N)}$ which was red-shifted relative to b R^{II}_{450} (480 vs 450 nm, respectively). Red-shifted photoproducts were generated in all of the above mutants following steady state illumination, except in the case of D85N. The latter observation demonstrates the critical role of the Asp-85 residue in generating the O_l species.

Light-induced pH measurements, analogous to those described above, were carried out in the case of $bR^{II}_{(D96A)}$ and $bR^{II}_{(E204Q)}$. We found that the kinetics of proton uptake and release were not significantly affected by the D96A mutation. This is in contrast to the behavior of $bR^{II}_{(E204Q)}$, as presented in Figure 9, which shows the thermal decay of the O_l species generated from $bR^{II}_{(E204Q)}$ and that of the basic form of the pyranine indicator. Whereas in bR^{II} and $bR^{II}_{(D96A)}$, the $\tau_{1/2}$ value of the dye signal is much faster than that of O_l (60 vs 300 s), the point mutation in Glu-204 delays the proton release reaction in such a manner that its rate coincides with that of the decay of O_l (220 s). Similar experiments were difficult to carry out in the case of $bR^{I}_{(E204Q)}$, since the fluorine-induced alteration of the protonated Schiff base pK_a leads to the formation of mixtures

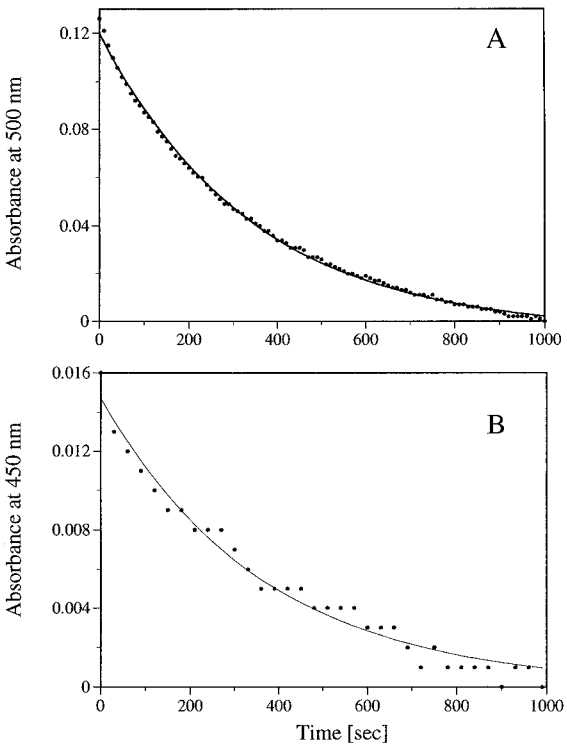


FIGURE 9: Thermal decays of (A) the photoproduct (λ_{max} = 500 nm) of pigment $bR^{II}_{(E204Q)}$ at pH 7.2 and (B) the basic form (λ_{max} = 450 nm) of pyranine under the same conditions. Solid lines are best-fit curves.

of O_l and of its deprotonated Schiff base. Interestingly, in R82Q mutants, no dye signals were detected following generation of the red-shifted photoproduct and its decay.

An analysis of the yields of the O^{I}_{640} and O^{II}_{510} photoproducts of the artificial pigments of D96A and R82Q indicates that the pK_a's associated with their formation are similar to those of the wild type; i.e. they are not affected by the respective mutations. In contrast, the pK_a was significantly affected by mutation at Glu-204 (Figure 10). As summarized in Table 2, the pK_a values were found to decrease to 7.0 and 6.9 in $bR^{II}_{(E204Q)}$ and $bR^{I}_{(E204Q)}$, respectively, whereas in bR^{I}_{588} and bR^{II}_{450}, the values were 9.0 and 8.7, respectively. The E204Q mutation was also found to affect the pK_a value of the PSB in O_l, decreasing it by approximately 2 units, to 9 and 7 in $bR^{II}_{(E204Q)}$ and $bR^{I}_{(E204Q)}$, respectively.

Transition to the Blue Membrane. Lowering the pH of the purple membrane (PM) of WT bR is known to be associated with a red shift in the absorption maximum, from 568 to 604 nm, due to the transition to the blue membrane (BM) form. The pK_a of the PM ⇌ BM transition (2.5 in 0.1 M NaCl) is assigned to the titration of Asp-85 (Metz et al., 1992). In order to detect the corresponding transition in pigments I−III, their dark-adapted forms were titrated in the region between pH 2 and 10. We found that acidification in this region generates two or three PM to BM transitions. Each transition is characterized by a specific pK_a value (see Table 3) and a characteristic purple to blue (BM minus PM) difference spectrum. These difference spectra differ in their λ_{max} values and in the relative absorbance.

The titration curves of pigments I and II (dark-adapted) in the presence of sodium salt (0.1 N NaCl) and the corresponding curve fits are shown in Figure 11. bR^{I} shows a transition with pK_a value of 3.8, whereas bR^{II} is characterized by a distinct transition with a pK_a value of 4.1. In water, both transitions are shifted up by approximately half a pK_a unit. We note that in bR^{II} a second transition probably exists around pH ∼8.5, as shown by the difference spectrum between pH 7 and 9 (trace B), which is different from that of trace A. It is important to note that the curves reflect behavior which is more complex than that of just two PM ⇌ BM transitions. Thus, the broad range of the pH change below pH 7 suggests the existence of more than one transition in this range (as shown for bR^{I} in the inset of Figure 11).

The titration curve of bR^{III} (Y185F mutant) in water (Figure 12A) consists of two clear transitions at pK_a's of 4.5 and 9.5. The maxima in the BM minus PM difference spectra in the region of pH 7−10 are 624 to 530 nm with a relative absorbance ratio of 1/1. Below pH 7, two types of conversions are evident: 614 to 530 nm with a relative absorbance ratio of 1/1 at pH 4−7 and 608 to 530 nm with a relative absorbance ratio of 2/1 at pH 2−4. Interestingly, the latter two transitions are well separated in the presence of sodium salt (0.1 N NaCl). Thus, as shown in Figure 12B, the curve titration is now characterized by two transitions with pK_a values of 2.7 and 4.0. In the presence of NaCl, no transition is detected in the pH 7−10 region.

As shown above, the pK_a of the formation of O_l in the E204Q mutant of pigments I and II is drastically altered. Since O_l formation is interpreted in terms of the protonation of Asp-85 (see Discussion below), we found it relevant to investigate the influence of this mutation on the pK_a of Asp-85 in the dark-adapted state. Titration of the dark-adapted form of $bR^{I}_{(E204Q)}$ (Figure 13, left) in the region below 4.8 results in a 676 to 580 nm (1/1) purple to blue transition difference spectrum (Figure 13, trace B). In the pH range between 6.6 and 4.8, a 670 nm absorbing species is induced by low pH without any noticeable depletion in the 580 nm region (trace A). The corresponding titration curve indicates that the pK_a values of the above two transitions are 5.3 and 4.0. The separation into such two transitions is detected in E204Q, even with the native retinal chromophore. The corresponding pK_a values are 4.2 and 2.6 (Figure 13, right). Finally, no transition is observed around pH 9 in $bR^{I}_{(E204Q)}$ and $bR^{II}_{(E204Q)}$ (data not shown).

DISCUSSION

Identification of the O_l Species. Several common features suggest that analogous O_l (O-like) species are formed upon light adaptation of bR that underwent a Tyr-185 point mutation, or of the artificial bRs in which the native retinal chromophore is replaced by chromophores 1 and 2. (a) The generation of all three O_l species is pH-dependent, exhibiting an equilibrium between O_l and a deprotonated purple form, with pK_a values that are similar in all three cases (see Table 2). (b) In all three cases, the photogeneration and thermal decay of O_l are accompanied by analogous proton uptake and release processes. (c) All three species are associated with an *all-trans* retinal configuration (Tierno et al., 1990; Steinberg et al., 1991; Rath et al., 1993). (d) Both O^{I}_{640} and O^{II}_{510} species are photoreversible upon illumination with red light. This feature has not been observed in the case of the Y185F mutant. At present, we are incapable of attributing this difference either to a different structural identity or to a

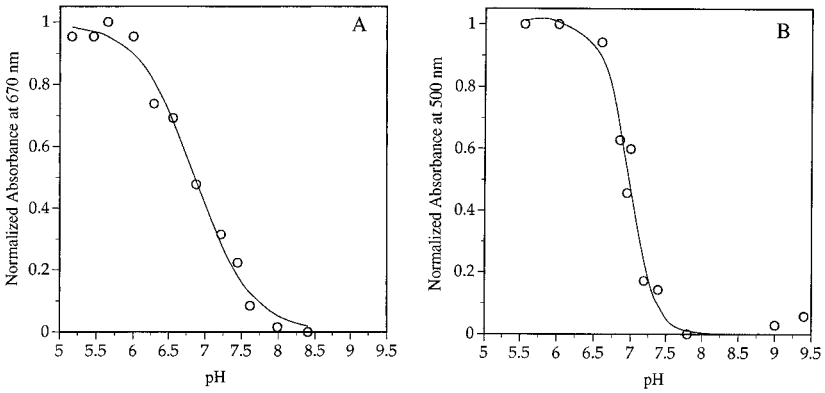


FIGURE 10: Titration curves of the red-shifted photoproduct of artificial E204Q pigments (10 mM NaCl): (A) E204Q derived from 14F retinal 1 and (B) E204Q derived from polyene 2. The solid line represents the best titration fit with a pK_a of 7.00 ± 0.03 (A) and a pK_a of 6.90 ± 0.05 (B).

Table 3: Apparent pK_a Values of the DA Form

pK_a of the Schiff base titration of the DA form			pK_a of the purple to blue transition of the DA form			
bR^{I}	bR^{II}	wild type	bR^{I}	bR^{II}	bR^{III}	wild type
11.5	12.8	13.3	3.8[b] 4.3[a]	4.1[b], ~8.5[b] 4.6[a]	2.7[b], 4.0[b] 4.5[a], 9.5[a]	2.5[b]
			$bR^{I}_{(E204Q)}$ 4.0[b], 5.3[b]			$bR_{(E204Q)}$ 4.2[b], 2.6[b]

[a] In water. [b] 0.1 M NaCl.

"technically" different photophysical behavior of O_l (e.g. quantum yield values, etc.).

We note that the Tyr-185 mutation, as well as the replacement of the native chromophores by the two synthetic molecules, caused a similar increase in the pK_a of the purple−blue transition of the corresponding DA pigments (see Table 2). This observation supports the assumption that in the three pigments similar structural and mechanistic changes in the protein were introduced.

Assuming that essentially similar O_l species are photogenerated in all three cases, the question arises as to the identification of O_l. In their pioneering series of papers that included a detailed vibrational analysis of O_l, Rothschild and co-workers (Sonar et al., 1993; Rath et al., 1993; He et al., 1993) identified O^{III}_{608} as the O intermediate of the photocycle of *all-trans*-bR^{III}_{550}. Thus, in variance with the O intermediate of WT bR, a substantial amount of O^{III}_{608} is already present in the dark in equilibrium with bR^{III}. This interpretation is consistent with the observation that illumination of O^{III}_{608} restores (via K and N intermediates) the original $O^{III}_{608} \rightleftharpoons bR^{III}_{550}$ equilibrium mixture (Sonar et al., 1993). However, this model is not applicable to pigments I and II in which O_l is photoreversible, yielding upon illumination purple species (bR^{I}_{588} and bR^{II}_{450}), which are identical to those obtained after the slow thermal decay (dark adaptation) of O^{I}_{640} and O^{II}_{510}, respectively. In other words, the purple species, which are in fast equilibrium with O_l, differ from bR^{I}_{588} and bR^{II}_{450} obtained by illumination of O_l.

An alternative identification of O^{III}_{608} (and thus possibly also of O^{I}_{640} and O^{II}_{510}), considered by Sonar et al., is that it may be the same as the acid-induced blue membrane of bR^{III}_{550}. This possibility assumes that the formation of O^{III}_{608} upon illumination is due to the 13-*cis* $\xrightarrow{h\nu}$ *all-trans* photo-

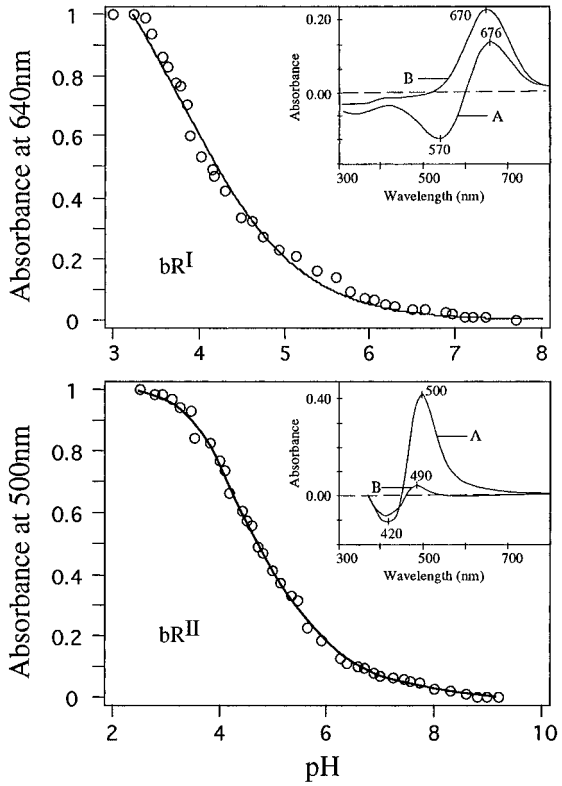


FIGURE 11: Titration of the purple to blue transition ([NaCl] = 0.1 M) of bR^{I}_{588} (upper) and bR^{II}_{450} (lower). The solid line represents the best titration fit with a pK_a of 3.8 ± 0.1 for bR^{I} and a pK_{a1} of 4.1 ± 0.1 and a pK_{a2} of ~8.5 for bR^{II}. (Inset) Absorption difference spectra at different pH ranges. For bR^{I}, A = absorption at pH 3 minus absorption at pH 4 and B = absorption at pH 4 minus absorption at pH 7. For bR^{II}, A = absorption at pH 3 minus absorption at pH 7 and B = absorption at pH 7 minus absorption at pH 9.

conversion as well as to a difference in the pK_a's of Asp-85 in the *all-trans* and 13-*cis* components. Thus, if the pK_a of *all-trans* is highly elevated in Y185F relative to that in WT bR, light adaptation will lead to an increase in the amount of the BM, i.e. of O_l. Since a considerable amount of *all-trans* isomer exists in the dark-adapted forms, at least in the case of bR^{I}_{588} and bR^{II}_{450} (Tierno et al., 1990; Steinberg et al., 1994), it will be implied that a substantial amount of blue membrane will be present in the dark, titratable with a pK_a of ~9. This was not observed in our experiments. Thus, we believe that this approach is highly unlikely. Another possibility is based on a model suggested by Balashov et al.

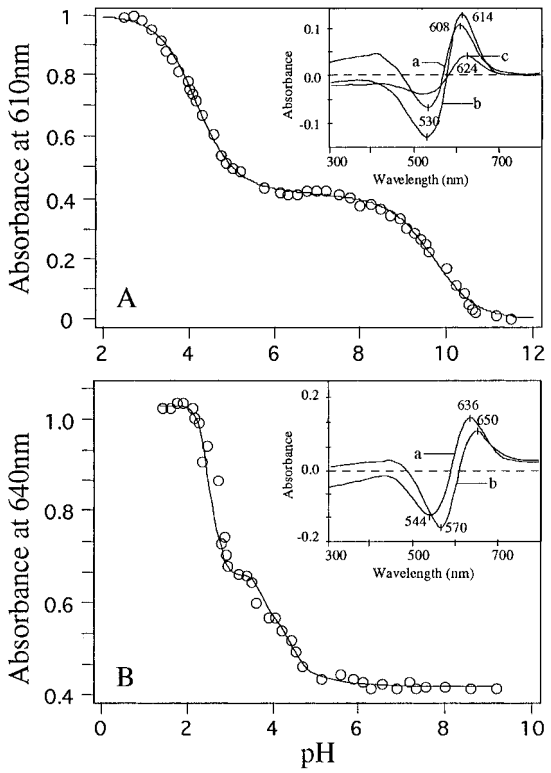
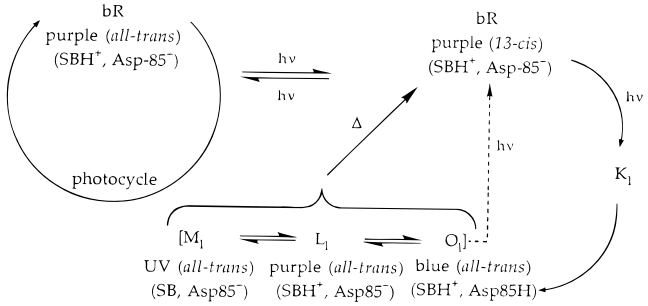


FIGURE 12: Titration of the purple to blue transition of bR^{III}: (A) In water without salt, with $pK_{a1} = 4.5 \pm 0.1$ and $pK_{a2} = 9.5 \pm 0.1$. (B) In the presence of 0.1 M NaCl, with $pK_{a1} = 2.7 \pm 0.1$ and $pK_{a2} = 4.0 \pm 0.1$. (Inset) Absorption difference spectra at different pH ranges. (A) a, absorption at pH 2.5 minus absorption at pH 4; b, absorption at pH 4 minus absorption at pH 7; and c, absorption at pH 7 minus absorption at pH 10. (B) a, absorption at pH 1.5 minus absorption at pH 3; and b, absorption at pH 3 minus absorption at pH 7.

(1995a,b, 1996) in which titration of Asp-85 is coupled to the titration of a second group denoted as X′H. The interactions between these two groups are described by four pK_a values, a situation which results in two observed (apparent) pK_a's for the titration of Asp-85. It was recently proposed that X′H is the group that releases the proton during the photocycle and that it should be identified as Glu-204 (Richter et al., 1996). Indeed, in the E204Q mutant, the titration of Asp-85 lacks the component around pH ~9, and furthermore, this component is lowered to a pK_a of ~8 in the E204D mutant. Balashov et al. (1996) also used this model to explain the observation that illumination of dark-adapted bR at pH ~3 increases the amount of the blue membrane, concluding that the *all-trans* and 13-*cis* isomers are characterized by a small (ca. 0.3 pK_a unit) difference in the pK_a's of Asp-85. Thus, the suggested interactions between X′H and Asp-85 and the different pK_a's of the *all-trans* and 13-*cis* isomers can, in principle, identify O_l as the blue membrane, since it requires a ca. 1 pK_a unit increase in the pK_a of the *all-trans* molecule relative to that of the 13-*cis* isomer. However, this model requires that the pK_a of Asp-85 in the blue membrane be affected markedly (a few pK_a units) by the protonation state of X′H, i.e. of Glu-204. This is not in keeping with our experiments (see detailed discussion below), which clearly demonstrate that the pK_a of Asp-85 in O_l is affected by only ca. 0.2 pK_a unit following deprotonation of Glu-204 (as detected by proton release to the medium).

In view of the inadequacy of the above approaches in identifying O_l, we present an alternative mechanism, which appears to be consistent with the accumulated experimental data. Accordingly, O_l is identified as a phototransient in the photocycle of the 13-*cis* (rather than *all-trans*) isomer. This identification, which is fully consistent with the behavior of pigments I and II, but is also applicable to Y185F, is based on the following Scheme 1:

Scheme 1



Accordingly, O_l is analogous to the red-shifted intermediate in the photocycle of the 13-*cis* form of native bR [previously denoted as L^c_{610} by Kalisky et al. (1977) and Lozier et al. (1978)]. This model assumes that the photocycles of 13-*cis*-bR^I, -bR^{II}, and -bR^{III} differ from that of WT bR in exhibiting a highly prolonged lifetime of the O_l intermediate. The accumulation of the O_l species as a relatively long-lived species is based on the fact that steady state illumination in the green drives all photoequilibria toward O_l, which is the longest-lived species of the two photocycles. Its exceptionally long lifetime enables the titration of O_l so that upon raising of the pH it converts to a purple form, denoted as L_l, and to a yellow form, denoted as M_l (see below for detailed discussion of these titrations). As in the case of O_l and O^I_{640}, the notations K_l, L_l, and M_l are used due to spectroscopical analogies but do not imply any structural identity with the K_{590}, L_{550}, and M_{412} intermediates of the photocycle of *all-trans* WT bR. It is relevant to note that, according to this interpretation, our data represent the first example of the titration of a photocycle intermediate in a retinal protein. Such titration, i.e. acid−base equilibrium of the Schiff base or of a protein residue with the extramembrane medium, is usually precluded by the relatively short lifetime of the respective intermediates [for a review, see Honig et al. (1995)]. This limitation is removed in the case of the O_l intermediates of pigments I, II, and possibly III, exhibiting lifetimes which are several orders of magnitude longer than that (50 ms) of O_l of the native pigment (Kalisky et al., 1977; Lozier et al., 1978)

It is important to note that the flash photolysis experiments of bR^I_{588} described above produced a red-shifted intermediate, which decays to its parent (purple) pigment with the same kinetics and difference spectrum as those observed for the O_l species produced by steady state illumination. This observation indicates that steady state illumination produced a genuine photointermediate of the primary photocycle (of the 13-*cis*-bR pigment), excluding the possibility that O_l is a secondary photoproduct produced by excitation of a primary photocycle intermediate.

The following major observations provide the basis of the proposed identification of O_l.

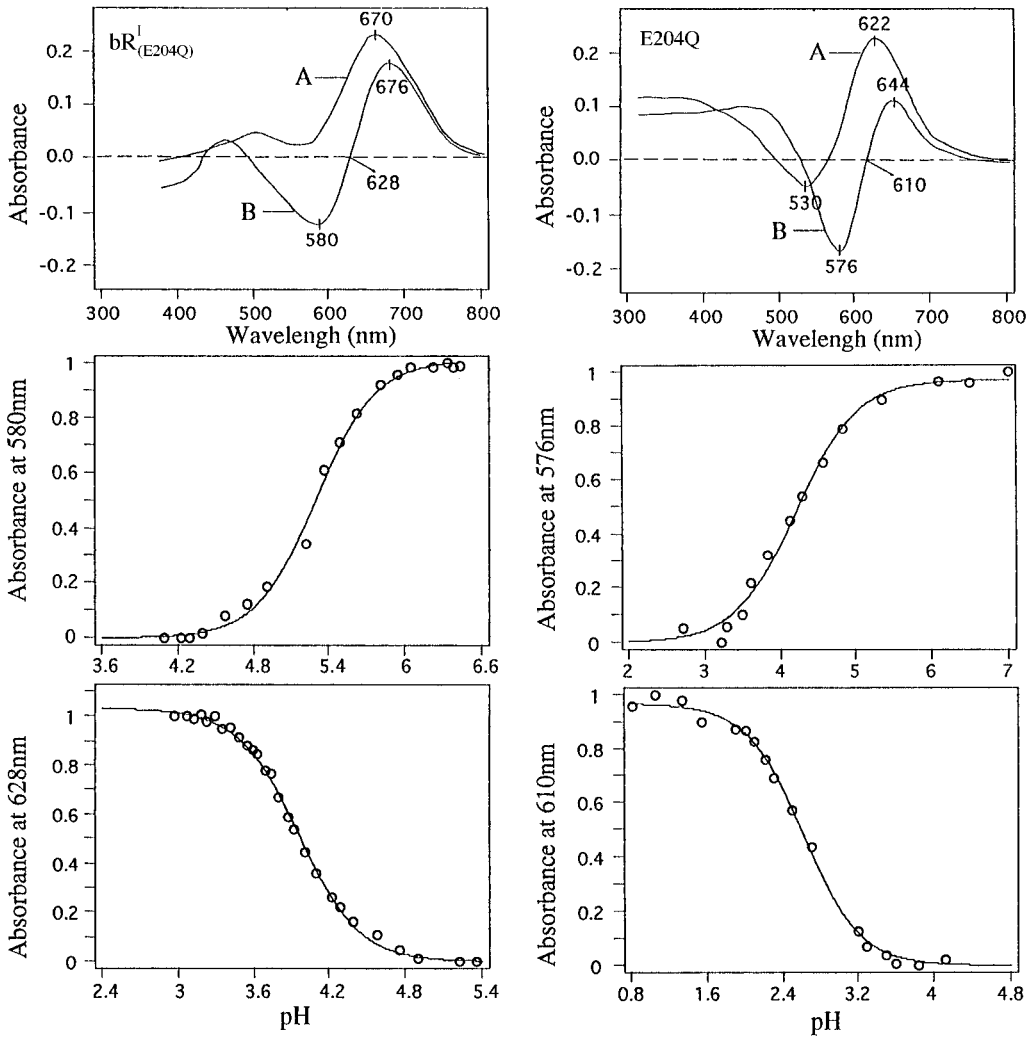


FIGURE 13: Titration of the purple to blue transition (with H_2SO_4) of the dark-adapted mutant E204Q (right) and E204Q derived from 14F retinal (left) in 0.1 M NaCl. The solid line represents the best titration fit with pK_{a1} = 4.2 and pK_{a2} = 2.6 (right) and pK_{a1} = 5.3 and pK_{a2} = 4.0 (left). We note that measurements at 580 or 576 nm represent more closely transition B and that measurements of 628 or 610 nm represent more closely transition A. The upper panels show absorption difference spectra at different pH values. (Right) A, absorption at pH 3.2 minus absorption at pH 7; and B, absorption at pH 0.8 minus absorption at pH 3.2. (Left) A, absorption at pH 4.4 minus absorption at pH 6; and B, absorption at pH 3 minus absorption at pH 4.4.

(a) The first is the *all-trans* structure of the chromophore in O_l. FT Raman and FTIR studies (He, 1993; Rath, 1993) have shown that the red-shifted photoproduct of Y185F is characterized by an *all-trans* chromophore. Similarly, chromophore extraction experiments in the artificial pigments bR^{I}_{580} and bR^{II}_{450} (Tierno et al., 1990; Steinberg et al., 1991) revealed an *all-trans* configuration for their red-shifted photoproducts, O^{I}_{640} and O^{II}_{510}. Furthermore, subsequent irradiation of O^{I}_{640} and O^{II}_{510} with red light reconverted them to a species absorbing in a manner similar to that of the parent pigment but is characterized by a 13-*cis* configuration. These experiments are in keeping with the assignment of O_l to a stable intermediate of the 13-*cis* photocycle, which is based on the13-*cis* → *all-trans* isomerization (see Scheme 1). As previously suggested (Tierno et al., 1990), the retinal structure in O_l may differ from that of the O_{640} intermediate of the *trans*-bR photocycle in its C=N configuration, *syn* vs *anti* correspondingly.

(b) The second is the protonated nature of Asp-85 in O_l and the associated proton release and uptake process. As shown in Scheme 1, we suggest that the red shift in the absorption maxima of the O^{I}_{640}, O^{II}_{510}, and O^{III}_{608} photoproducts is due to protonation of Asp-85. Accordingly, the blue shift observed for these photoproducts following pH elevation is due to deprotonation of Asp-85. This suggestion is based on the following observations. (1) The transition from the parent pigments to the low-pH BM forms is associated as well with a red shift with a magnitude comparable to that observed for the O^{I}_{640}, O^{II}_{510}, and O^{I}_{608} photoproducts. It is well accepted that in wild-type bR the PM to BM transition is due to Asp-85 protonation (Metz et al., 1992). (2) FTIR studies (He et al., 1993) of the red-shifted photoproduct in the Y185F mutant revealed that this species exhibits vibrational bands, which are similar to those of the O intermediate of the *all-trans* photocycle, including the protonated state of Asp-85. If, as discussed above, the O^{I}_{640} and O^{II}_{510} photoproducts of the artificial pigments bR^{I} and bR^{II} are structurally identical to O^{III}_{608}, it is reasonable to assume that they are characterized by a protonated Asp-85 as well. (3) In a manner analogous to that of the mutant, D85N which exhibits a red shift in the spectrum relative to that of native bR, due to replacement of the negatively charged Asp-85 by the neutral residue aspargine, $bR^{II}_{(D85N)}$, absorbs at 480 nm whereas WT bR^{II}_{450} absorbs at 450 nm. However,

in keeping with the lack of a titratable Asp-85 counterion, this artificial pigment is the only one which upon irradiation does not form any red-shifted O_l photoproduct. (4) The H^+ uptake process, which accompanies the formation of O_l, is consistent with a protonation reaction.

(c) The third is the photoreversibility of O_l. In the case of pigments I and II, the corresponding O_l species are reconverted by red light to the respective purple dark-adapted forms, bR^{I}_{588} and bR^{II}_{450}. In this respect, O^{I}_{640} and O^{II}_{510} behave in a manner identical to that of the O_l photointermediate of the 13-*cis* isomer of native bR (previously denoted as L^{c}_{610}), which was shown to back photoreact to 13-*cis*-bR (Lozier et al., 1978). As mentioned above, photoreversibility was not detected in the case of O^{III}_{608}. Since photoreversibility is an important element in our suggested identification of O^{I}_{640} and O^{II}_{510}, its absence in the case of O^{III}_{608} may suggest that the latter is structurally different. In other words, if the lack of reversibility of O^{III}_{608} is not due to technical reasons (e.g. similar quantum yields of both reactions in the irradiated wavelength), its identification as being analogous to the O^{I}_{640} photointermediate of the *all-trans* photocycle, as suggested by Sonar et al. (1993), should be retained.

pK_a of Asp-85 in the O_l Species. An important observation is that in the O_l species the pK_a of Asp-85 is considerably elevated (to ca. 9) with respect to that of the PM to BM transition (pK_a = 2.5−4.5). Moreover, the PSB pK_a in O^{I}_{640} and O^{II}_{510} is also affected, being decreased by 2 units relative to those of the parent pigments, as well as to that of native bR (Druckmann et al., 1982; Sheves et al., 1988). These observations may be rationalized in terms of studies with model compounds showing that a specific angle between the PSB and the carboxyl counterion allows bound water molecules to form a defined structure and to bridge the two groups (Gat & Sheves, 1993). The defined bound water structure causes an effective stabilization of the ion pair, inducing a high pK_a for the PSB and a relatively low one for Asp-85. The importance of bound water in controlling the pK_a of these residues was recently supported by theoretical calculations (Sampogna & Honig, 1994). In addition, the role that the geometry of the proton donor and acceptor plays in controlling proton transfer in the photocycle was later demonstrated by studies with bR mutants (Brown et al., 1994). The major role that geometrical arrangements play in controlling the pK_a of the ion pair was further analyzed by using artificial pigments (Rousso et al., 1995). It was shown that different conformations in the bR binding site imposed by different retinal structures alter considerably the pK_a of both the PSB and Asp-85. We suggest that the well-organized hydrogen bonding network which is mediated through bound water in the parent pigments I−III is disturbed in their O_l photoproduct, altering the pK_a of both PSB and Asp-85 groups. Thus, the pK_a of Asp-85 increases by 6 units, causing its protonation at neutral pH, whereas the effect on the PSB pK_a is smaller and is insufficient for inducing deprotonation. Thus, the formation of O_l should be accompanied by a net proton uptake from the aqueous medium by Asp-85, in keeping with our experimental observations. The higher sensitivity of the pK_a of Asp-85 with respect to that of the PSB, to environmental changes in structure, is in keeping with a recent analysis based on artificial pigments (Rousso et al., 1995) as well as with theoretical calculations (Sampogna & Honig, 1994).

It has been shown that in bR the pK_a of Asp-85 is considerably affected by the mutation of Arg-82, being elevated from 2.5 to 7.5 in both R82A and R82Q mutants (Subramaniam et al., 1990; Otto et al., 1990; Thorgeirsson et al., 1991; Brown et al., 1993; Balashov et al., 1993). It was suggested that this effect is due to electrostatic interactions between Asp-85 and Arg-82 due to their proximity. Interestingly, in O^{I}_{640} and O^{II}_{510}, the pK_a of Asp-85 is not affected by the Arg-82 mutation. This might point to the possibility that in O_l residues Arg-82 and Asp-85 are not in close proximity, i.e. that their interaction is considerably altered during the 13-*cis* photocycle. In contrast, we have shown that in O_l the pK_a of Asp-85 as well as that of the PSB are considerably affected by the E204Q mutation, being decreased to 7 and 9, respectively. As discussed below, Glu-204 appears to be protonated in the early phase of O_l formation, indicating that the mutation did not alter the uncharged state of the Glu-204 residue. We are, therefore, led to conclude that the effect of 204 mutation on the pK_a of both Asp-85 and the Schiff base in O_l is through alteration of a hydrogen bonding network, probably involving structured water, and not through charge interactions. This effect is in keeping with our suggestion (Gat & Sheves, 1993; Rousso et al., 1995) that the pK_a of protein residues, and proton transfer between them, is markedly affected by the geometrical arrangements of structured water that bridge these residues.

Proton Uptake and Release Activity. Time-resolved titrations of unphotolyzed bR led to the conclusion that Asp-85 is exposed to the extracellular side (Druckmann et al., 1982, 1995; Brown et al., 1994). The observation that proton uptake following the formation of the O-like species of pigments I and II is not affected by the D96A mutation indicates that Asp-96 may not be involved in the proton uptake process. Since Asp-96 is exposed to the cytoplasmic side, this implies that, as in the case of an unphotolyzed pigment, the exposure of Asp-85 also in O_l is to the outside.

An important observation is associated with the proton release kinetics, as implied by the decay of the dye signal. We have shown that proton release is much faster than the thermal decay of the O-like intermediate. This behavior is absent in the E204Q artificial pigment I, in which proton release is delayed so that both processes, proton release and O_l decay, are characterized by matching kinetics. We propose that in the 13-*cis* photocycle proton uptake by Asp-85, which leads to O_l, is followed by a proton release from Glu-204 (or from another group affected by the Glu-204 mutation). Accordingly, the absence of Glu-204 delays the proton release process, which may then take place only upon the decay of O_l, namely when Asp-85 itself releases its proton directly to the outside. Recent studies identify Glu-204 as the proton-releasing group in the photocyle of *all-trans* wild-type bR (Brown et al., 1995). Thus, in both 13-*cis* and *all-trans* systems, protonation of Asp-85 leads to deprotonation of Glu-204 (or of a protein residue associated with Glu-204). When the latter is replaced by a nondeprotonatable group, proton release occurs directly from Asp-85 on a much slower time scale.

A question, which is fundamental to the understanding of the *all-trans*-bR photocycle, concerns the mechanism by which a proton is released following protonation of Asp-85.

Electron diffraction studies revealed that Glu-204 is located close to the extracellular surface in the proximity of Asp-85 (Henderson et al., 1990; Grigorieff et al., 1996). Thus, a plausible mechanism is that neutralization of Asp-85, via proton uptake, reduces the pK_a of Glu-204, due to changes in electrostatic interactions, leading to its deprotonation. Recent FTIR studies (Brown et al., 1995) have suggested that Glu-204 is protonated in unphotolyzed bR and undergoes deprotonation in the M state. As the source of the released protons, Glu-204 was suggested to have a high pK_a in the unphotolyzed protein which decreases to about 6 during the photocycle. Molecular dynamics simulations (Humphrey et al., 1994) suggest a chain of three hydrogen-bonded water molecules connecting Glu-204 to Arg-82, which directly participate in the counterion complex to the Schiff base. In fact, in the case of the *all-trans* isomer, molecular dynamics and electrostatic calculations (Scharnagl et al., 1995) were used to estimate a pK_a reduction for Glu-204 from 13 before photoexcitation to 6−8 in the M state, in keeping with the fast deprotonation of this residue during the photocycle. However, the present results show that the half-time of the proton release process in the 13-*cis* photocycle of pigments I−III occurs on a time scale of seconds. This large delay (with respect to proton uptake) suggests that protonation of Asp-85 is not sufficient for proton release to occur and that an additional process after Asp-85 protonation is necessary for releasing the proton in the 13-*cis* photocycle and possibly also in the *all-trans* photocycle. We note that it was previously shown that in the mutant D85E protonation of E85 by the protonated Schiff base, following formation of the M intermediate in the *all-trans* photocycle, is not the rate-limiting step for the proton release cascade (Herberle et al., 1993). Thus, besides Asp-85 protonation, an additional protein change must occur that induces reduction of the pK_a of that proton-releasing group and/or opens a channel to release the proton. We suggest that this process involves rearrangement of the hydrogen bonding network, including bound water, which decreases the pK_a of the relevant group. This implies that the pK_a of the group, which is the source of the released proton, is controlled by H bonds rather than by simple electrostatic interactions with Asp-85. This suggestion is also supported by the result, that, although upon E204Q mutation the uncharged state of the 204 residue is not affected, a significant alteration of the pK_a of Asp-85 and the protonated Schiff base in O_l photoproduct is observed.

Another possible cause for the delay in proton release following protonation of Asp-85 may be associated with a conformational change which involves residue Arg-82. The location of this residue in the electron diffraction model (Henderson et al., 1990; Grigorieff et al., 1996) is ambiguous, but molecular dynamics models (Humphrey et al., 1994; Scharnagl et al., 1995) position Arg-82 between Asp-85 and Glu-204. This theoretical evidence suggests (Scharnagl et al., 1995) that following protonation of Asp-85, which decreases the electrostatic interactions between Asp-85 and Arg-82, the latter switches from the "in" to the "out" conformation and moves away from Asp-85. This movement, which decouples these two residues, leads to an increased electrostatic interaction between Arg-82 and Glu-204 which is suggested to induce the reduction in the pK_a of Glu-204. This conformational change is in keeping with the observations that in the unphotolyzed R82Q mutant the pK_a of Asp-85 is significantly altered and that in the R82Q photocycle proton release is inhibited due to insufficient reduction of the proton-releasing group in the M intermediate (Govindjee et al., 1996). In this work, we have shown that this mutation does not affect the pK_a of Asp-85 in the O-like intermediate. This is consistent with a decoupling of Arg-82 and Asp-85 in O_l, analogous to the proposed situation in the WT *all-trans* photocycle.

As described above, the generation of O_l is not affected by the R82Q mutation, but no proton uptake and release reactions were detected in this system. The latter phenomenon may be accounted for in terms of two possible explanations. (1) The kinetics of the proton release are accelerated to a time scale comparable to that of the proton uptake process. (2) An internal proton uptake and release takes place without involving the aqueous bulk.

An important feature of the O-like intermediate is the resulting proton release, with identical kinetics, at both pH 7 and 5. This implies that the pK_a of the proton-releasing group in O_l is lower than ∼4.5. It was found (Zimanyi et al., 1992) that, in the bR photocycle at pH <6, proton release is delayed until the last step of the photocycle. The effect was attributed to a pK_a value of about 6 for the proton-releasing group (XH). Under these low-pH conditions, the proton is released (presumably directly from Asp-85) during the thermal decay of the O_{640} intermediate. Despite the fact that the O-like intermediate observed in the present studies is suggested to be associated with the 13-*cis* photocycle, we may assume similar features for O_l and O_{640}. In such a case, it may be suggested that the pK_a of the proton-releasing group is reduced to about 6 in the first half of the photocycle (i.e. up to the M_{410} stage) but is further reduced (to <4.5) in the O_{640} intermediate. Furthermore, it may suggest that, in the *all-trans* photocycle at pH <6, the proton is released during the O_{640} intermediate lifetime from the XH group and not directly from Asp-85.

pK_a *of the PSB in* O_l. As shown in Figure 8 and Table 2, the pK_a of the protonated Schiff base in O^{I}_{640} and O^{II}_{510} is decreased by about 2 units relative to those of the parent pigments. This implies that, although the state of Asp-85 in these O-like intermediates is neutral (i.e. the interaction with carboxylate-85 is avoided), the PSB pK_a is reduced by 2 units, in contrast to the situation prevailing in the unphotolyzed D85N mutant, where replacement of carboxylate-85 with an amide reduces the PSB pK_a by 5−6 units, from 13.3 to 7.0 or 8.4 (Otto et al., 1990; Brown et al., 1993), and to the situation where halorhodopsin lacks an anionic residue equivalent to D85 in which the PSB pK_a is 7.9 (without anions; Varo et al., 1986). In addition, in contrast to the behavior of WT bR, the pK_a of the PSB in O^{I}_{640} and O^{II}_{510} is revealed to be affected by E204Q. Thus, replacement of the glutamic acid by glutamine reduces the pK_a by 2 units, whereas no change in the pK_a is observed in the parent pigments. The observations may be rationalized by assuming that the postulated network of hydrogen-bonded water molecules involving the PSB, Asp-85, and Glu-204 is markedly altered in the O_l state. Consequently, the pK_a of the PSB, and its sensitivity to the protonation states of Asp-85 and Glu-204, are markedly affected with respect to the dark-adapted pigment. It was recently proposed that the pK_a of the retinal protonated Schiff base during the retinal Schiff reprotonation in the bR photocycle is 8.2−8.3 (Brown &

Lanyi, 1996). Our results suggest that the pK_a in the O intermediate is further increased by ca. 3 pK_a units.

pK_a *of the Purple to Blue Transition*. The purple to blue transition of pigment III, and probably also II, is characterized by, besides the low-pH transition, one around pH 9 (Table 3). Analogous behavior is observed in the case of WT bR and has been interpreted by assuming that Asp-85 interacts with another protein group, probably Glu-204 (Balashov et al., 1993, 1995, Richter et al., 1996), inducing a biphasicity in the Asp-85 titration. The titrations around pH 9 observed for pigments II and III are in keeping with this model. In fact, this transition disappears in pigment II derived from the E204Q mutant, which supports the possibility that in the unphotolyzed pigment Asp-85 is influenced by the titration of Glu-204. We note that, in the Y185F mutant, this transition disappears in the presence of salt. The underlying cause for this phenomenon is still unclear.

An interesting observation is that the low-pH component (below pH 7) of the purple to blue transition of pigments I−III clearly reflects two superimposed transitions. In the E204Q mutants of pigments I and II, these transitions are well separated, being characterized by pK_a's differing by 1.3 and 1.2 units, respectively (Table 3). These transitions also differ in their PM minus BM difference spectra. Because they are unaffected by dark−light adaptation, it is highly unlikely that the two transition components originate from different isomeric forms, i.e. *all-trans* and 13-*cis*. It is more reasonable that another protein residue, with a pK_a close to that of Asp-85, is titrated, affecting the titration of Asp-85. Future studies should identify this group.

Stability of O_l *and its Isomeric Configuration*. The exact reason for the unusual thermal stability of the O_l species in pigments I−III is still unclear. This phenomenon should be considered in view of the late stage of the *all-trans* photocycle, involving the N ⇌ O ⇌ bR transitions. These transformations include 13-*cis* → *all-trans* isomerization of the retinal chromophore and proton transfer from Asp-85 to the proton-releasing group (XH). Thermal isomerization is controlled by π-electron delocalization along the polyene skeleton, and as shown by model compound studies (Baasov & Sheves, 1984) and by theoretical calculations (Warshel, 1978), it is catalyzed by weak electrostatic interactions between the protonated Schiff base and its counterion. In keeping with this, it was recently shown that the dark adaptation process is highly catalyzed by protonation of Asp-85, which probably enhances charge delocalization (Balashov et al., 1993). A similar mechanism appears to control the 13-*cis* → *all-trans* relaxation in the photocycle at the O_{640} stage where (as indicated by the red-shifted absorption) the retinal chromophore experiences a high level of π-electron delocalization. However, even this highly delocalized species is characterized by slow isomerization if steric interactions between the chromophore and the protein are perturbed. This appears to occur in the case of the L93A mutant where the O_{640} intermediate accumulates due to inhibition of its decay. It was shown that in this case the O_{640} state is characterized by a 13-*cis* configuration and its thermal decay is slowed since its reisomerization to *all-trans* is inhibited by specific protein−chromophore interactions (Delaney et al., 1995).

Analogous perturbations may be responsible for the long lifetime of O_l. Thus, as suggested above, a common feature of the three pigments studied is perturbation of the hydrogen bonding network in the binding site, either by Y185F mutation or by introducing specific geometrical pertubations in the retinal skeleton, either by fluorine substitution at the C_{l4} position or by eliminating the β-ionone ring. We suggest, therefore, that thermal reisomerization is rate-determining in the decay of O_l and is markedly inhibited by such structural perturbations. This is also in keeping with the occurrence of the O_l back photoreaction, in which thermal isomerization is replaced by a photochemical process. To conclude, specific steric interactions between the chromophore and the protein play a major role in controlling the thermal isomerization, in either *cis* or *trans* photocycle. Steric interactions probably impose further barriers relative to model compounds in solution in the unphotolyzed pigment and reduce barriers in the O intermediate.

CONCLUSIONS

The study of artificial bR pigments derived from synthetic retinals, or of bR mutants with selectively modified protein residues, provides powerful tools for elucidating structural and mechanistic aspects of the function of this intriguing photosynthetic system. In the present work, we have applied these methodologies (separately), as well as a combination of both, to clarify the structure and function of the red-shifted (O) intermediate in the photocycle of 13-*cis*-bR. It is evident that the structural changes imposed in the retinal, and/or its protein environment, have modified the photocycle in a way which allows the titration of the two key residues, Asp-85 and the PSB, during the lifetime of the O_l phototransient. The results bear on basic features of proton release and uptake in the photocycles of both 13-*cis*- and *all-trans*-bR. A major conclusion concerns the role of the structured water network in the chromophore binding site in controlling the decay of the O intermediates (via *cis* → *trans* or *trans* → *cis* isomerization) of both bR photocycles.

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