On the Protein Residues that Control the Yield and Kinetics of O_{630} in the Photocycle of Bacteriorhodopsin
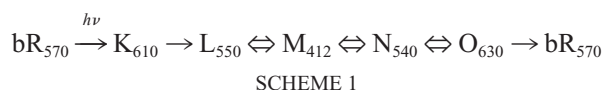
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ABSTRACT   The effects of pH on the yield (ϕ_r), and on the apparent rise and decay constants (k_r, k_d), of the O_{630} intermediate are important features of the bacteriorhodopsin (bR) photocycle. The effects are associated with three titration-like transitions: 1) A drop in k_r, k_d, and ϕ_r at high pH [$pK_a(1) \sim 8$]; 2) A rise in ϕ_r at low pH [$pK_a(2) \sim 4.5$]; and 3) A drop in k_r and k_d at low pH [$pK_a(3) \sim 4.5$]. (pK_a values are for native bR in 100 mM NaCl). Clarification of these effects is approached by studying the pH dependence of ϕ_r, k_r, and k_d in native and acetylated bR, and in its D96N and R82Q mutants. The D96N experiments were carried out in the presence of small amounts of the weak acids, azide, nitrite, and thiocyanate. Analysis of the mutant's data leads to the identification of the protein residue (R_1) whose state of protonation controls the magnitude of ϕ_r, k_r, and k_d at high pH, as Asp-96. Acetylation of bR modifies the Lys-129 residue, which is known to affect the pK_a of the group (XH), which releases the proton to the membrane exterior during the photocycle. The effects of acetylation on the O_{630} parameters reveal that the low-pH titrations should be ascribed to two additional protein residues R_2 and R_3. R_2 affects the rise of ϕ_r at low pH, whereas the state of protonation of R_3 affects both k_r and k_d. Our data confirm a previous suggestion that R_3 should be identified as the proton release moiety (XH). A clear identification of R_2, including its possible identity with R_3, remains open.

INTRODUCTION

The light-induced proton pump, bacteriorhodopsin (bR), is associated with a series of spectroscopic transformations represented by the simplified photocycle,

$$bR_{570} \xrightarrow{h\nu} K_{610} \rightarrow L_{550} \Leftrightarrow M_{412} \Leftrightarrow N_{540} \Leftrightarrow O_{630} \rightarrow bR_{570}$$

SCHEME 1

where subscripts refer to maximum absorption wavelengths. (See Ottolenghi and Sheves, 1995, and Lanyi and Váró, 1995, for a series of review articles on retinal proteins and bR, respectively.) The primary consequence of light absorption is the isomerization of the retinal chromophore, from *all-trans* in bR_{570} to *13-cis* in K_{610}. During the $L_{550} \rightarrow M_{412}$ transition, an intramembrane proton transfer takes place from the protonated Schiff base, which links the retinal chromophore to the protein, to the neighboring Asp-85 residue. Concurrently, at neutral pH, a proton is released to the extracellular side by a proton releasing moiety (XH) which most probably involves several amino acids (primarily, Glu-204 and Glu-194) as well as water molecules (Ottolenghi and Sheves 1995; Brown et al., 1995; Rammelsberg et al., 1998; Balashov et al., 1997; Dioumaev et al., 1998). The subsequent $M_{412} \rightarrow N_{540}$ step involves reprotonation of the Schiff base by Asp-96, which is closer to the cytoplasmic side. The vectorial proton translocation is completed when Asp-96 regains a proton from the cytoplasmic side, parallel to *13-cis* $\rightarrow$ *all-trans* isomerization of the

chromophore (the $N_{540} \rightarrow O_{630}$ transition). The initial state of the system is regained during the last $O_{630} \rightarrow bR_{570}$ step, as Asp-85 reprotonates the proton release group X^-. At low pH, when XH ($pK_a \cong 5.8$) is deprotonated, the proton release step is delayed to the last, $O_{630} \rightarrow bR_{570}$, stage as it occurs from Asp-85 to the extracellular medium (Zimanyi et al., 1992). It should be noted that, although most of this picture is generally accepted, the detailed molecular scheme of the bR photocycle is still an open problem. For example, findings that could not be explained by the sequential Scheme 1 were interpreted in terms of parallel (branching) photocycles (see review by Lanyi and Váró, 1995).

Due to the key role that O_{630} plays in the last phase of the pump mechanism, considerable attention has been given to the marked pH dependence of the relative amounts of O_{630} (here denoted as ϕ_r) and N_{540}. Upon decreasing the pH, ϕ_r increases, exhibiting a two-stage titration-like curve with pK_a values (in 0.1 M salt) of $pK_a(1) = 7.5–8$, (Lozier et al., 1978; Li et al., 1984; Ames et al., 1990; Eisfeld et al., 1993; Cao et al., 1993a; Chernavskii et al., 1989; Souvignier and Gerwert, 1992; Misra et al., 1997; Bressler et al., 1999; Balashov et al., 1999) and $pK_a(2) = 3–4$ (Bressler et al., 1999; Balashov et al., 1999), respectively. Analogous pH effects were reported for the apparent rate constants of O_{630} rise (k_r) and decay (k_d). Thus, k_d was shown to decrease both at high pH [$pK_a(1) = 7.5–8$], and at low pH [$pK_a(3) = 4.5$] (Eisfeld et al., 1993; Cao et al., 1993a; Bressler et al., 1999; Balashov et al., 1999). As to k_r, in addition to a low pH titration analogous to that of k_d, (Eisfeld et al., 1993; Cao et al., 1993; Bressler et al., 1999; Balashov et al., 1999), a drop at high pH ($pK_a = 7.5–8$) was reported (Eisfeld et al., 1993; Bressler et al., 1999). On the basis of studies carried out with mutants of Glu-204 (Bressler et al., 1999; Balashov et al., 1999) and Glu-194 (Balashov et al., 1999), the pH

effects on ϕ_r, k_r, and k_d have recently been interpreted in terms of the titration of several protein residues. Three residues were suggested by Bressler et al. (1999): R_1 [pK$_a$(1) $\cong$ 8.0], R_2 [pK$_a$(2) > 3.1], and R_3 [pK$_a$(3) = 4.6], the corresponding pK$_a$ values referring to 0.1-M NaCl solutions. An alternative approach (Balashov et al., 1999) identifies R_2 and R_3 as the same residue, specifically, as the proton release group XH (pK$_a$ = 4.3). As plausible candidates for the high pK$_a$(1) transition, Asp-96 (Balashov et al., 1999) and Arg-82 (Bressler et al., 1999) were suggested.

In the present work we test these models by investigating the effects of pH on the yields and kinetics of the O$_{630}$ intermediate in the R82Q and D96N mutants, the latter in the presence of several weak acids. Analysis of the data excludes Arg-82, and identifies Asp-96 as the residue (R_1) that controls the relative amount, and the apparent rise and decay constants, of O$_{630}$ at neutral and high pH. Experiments with bR, carrying an acetylated Lys-129 residue, are carried out based on previous information that Lys-129 affects the pK$_a$ of the proton release group during the photocycle (Govindjee et al., 1997). These data confirm the identification of R_3 as XH, but leave open that of R_2, in keeping with the earlier suggestion (Bressler et al., 1999) that pK$_a$(2) and pK$_a$(3) may correspond to different protein residues.

MATERIALS AND METHODS

Membrane suspensions of light-adapted native (wild-type, WT) bR (bR = 10^{-5} M), acetylated bR (Ac-bR), and of the R82Q and D96N mutants were exposed to 532-nm, 9-ns pulses of a Nd:YAG laser. Light-induced absorbance changes in light-adapted samples were recorded using a continuous 75 W xenon lamp, a photomultiplier, and a TDS Tektronix digitizer. Data were averaged and analyzed, using a personal computer.

To avoid aggregation at low pH (below pH ~ 3.0), the bR membranes were enclosed in polyacrylamide gels, following the procedure of Mowery et al. (1979). In these samples, the pH was adjusted by incubating the gels in 0.05 M citrate-phosphate-borate buffer for one hour. Acetylation was carried out according to a modified procedure of Takeuchi et al. (1981). An acetic anhydride solution was added in six 5-μl portions, with 5 min intervals, to an ice-cooled 5-ml membrane suspension of 5 × 10^{-5} M bR in 2.5 M sodium-acetate solution. The solution was stirred for one hour, and its pH was monitored and controlled (at pH 7.5) by the addition of NaOH. Final phenol hydrolysis was performed by stirring at 0°C at pH 9.2 for one hour, followed by water dilution and centrifugation. Pyranine studies were carried out according to Steinberg et al., 1994.

RESULTS AND DISCUSSION

R82Q

The replacement of Arg-82 with uncharged residues, such as in the mutants R82Q and R82A, markedly affects the pump mechanism, as well as the pK$_a$ of Asp-85 (the Purple ⇔ Blue equilibrium) (Balashov et al., 1993; Brown et al., 1993; Govindjee et al., 1996). Although, in these mutants, essentially no O$_{630}$ accumulates at room temperature (Cao et al., 1995; Otto et al., 1990; Duñach et al., 1990; Thor-

geirsson et al., 1991), substantial O$_{630}$ amounts were observed (at neutral pH) at higher temperatures (Cao et al., 1993a). Consequently, we have measured the relative yield and the kinetics of O$_{630}$ formation and decay over the 6.4 < pH < 8.5 range in R82Q membrane suspensions at 45°C in the presence of 2 M NaCl. Experiments at lower pH values were precluded by the substantial conversion into the blue form, due to the high pK$_a$ of Asp-85 in this mutant. (Note that the higher temperature reduces the pK$_a$ value of the Purple ⇔ Blue transition in the R82Q mutant and allows for O$_{630}$ tracking at pH values as low as 6.5).

In our previous work, we derived ϕ_r (the ratio between the measured O$_{630}$ amplitude and the maximum attainable amplitude) from the observed maxima of the transient absorbance traces at 670 nm, after correcting for the effects of the L$_{550}$ ⇔ M$_{412}$ and Purple ⇔ Blue equilibria at low pH (Bressler et al., 1999). The apparent rise and decay constants of O$_{630}$, k_r and k_d, were estimated from the half lives of the rise and decay of the corresponding traces, τ_r and τ_d, respectively. This approach is strictly valid when $\tau_r \ll \tau_d$, whereas, in these studies, $\tau_d = (6-10)\tau_r$. In the present work, we improved our analysis by simulating the time dependence of the relative amount of O$_{630}$ using numerical solutions to the two-step sequence,

$$(\text{L}_{550}, \text{M}_{412}, \text{N}_{540}) \xrightarrow{k_r} (\text{O}_{630}) \xrightarrow{k_d} \text{bR}.$$

SCHEME 2
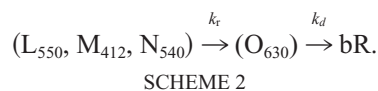
The three parameters, ϕ_r, k_r, and k_d, obtained from the simulations, are plotted in Fig. 1. It is evident that a high pK$_a$ titration, analogous to that of the native chromophore, attributed to residue R_1 (see Bressler et al.(1999) and Figs. 2 and 4 below), is present in the R82Q mutant. The corresponding pK$_a$ values (see Table 1) were derived by fitting to the single titration equation $A(\text{pH}) = B + C_1/(1 + 10^{(\text{pKa-pH})})$. $A(\text{pH})$ is the calculated rate (k_r or k_d) or yield (ϕ_r) parameter, C_1 is the amplitude of the respective titration, and B is a constant. Analogously to WT bR, a common apparent pK$_a$ value of $\cong$7) is obtained for all three titrations. It is thus evident that, although down-shifted by about one pK$_a$ unit with respect to the WT at room temperature (see Table 1), the pK$_a$(1) titration is maintained in the R82Q mutant. The fact that the mutation has similarly affected the three titrations confirms our working hypothesis (Bressler et al., 1999), which attributes them to the same residue, R_1. We conclude that R_1 cannot be identified with Arg-82. We note that, due to the relatively high pK$_a$ of Asp-85 in R82Q, the effect of this mutation on the low pH titrations, pK$_a$(2) and pK$_a$(3), could not be investigated.

D96N

Replacement of Asp-96 by uncharged residues results in a slow and pH-dependent M$_{412}$ decay due to the retardation of the Schiff base reprotonation (Holtz et al., 1989; Miller et
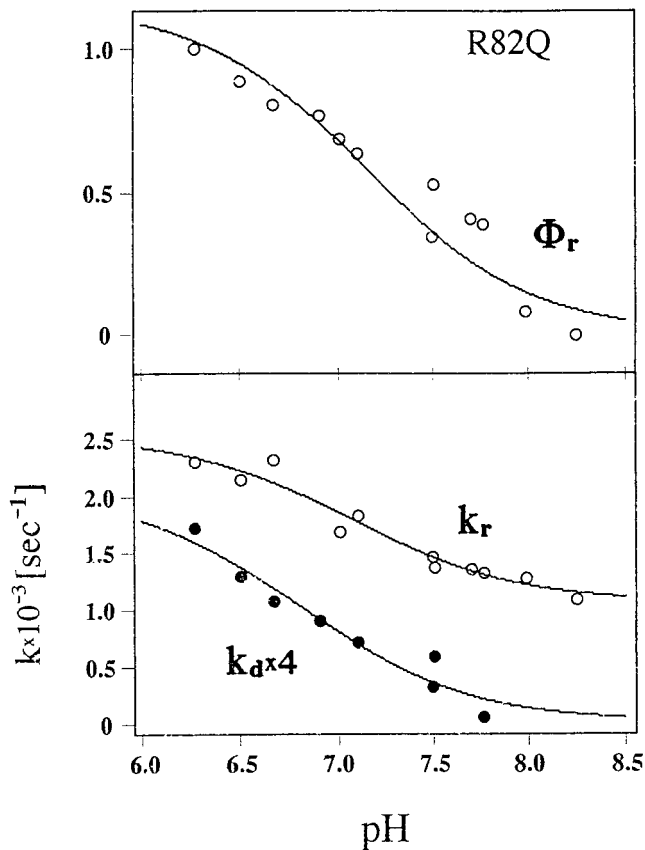
FIGURE 1 pH effects on the O_{630} parameters, ϕ_r, k_r, and k_d in the R82Q mutant, at 45°C, in the presence of 2 M NaCl. Continuous lines are calculated titration curves, fitted as described in the text.

al., 1990). Under such conditions, O_{630} is not accumulated. However, as shown by Tittor et al. (1989), small anions of weak acids, such as cyanate, azide, nitrite, formate, and

acetate, fully restore the basic photocycle kinetics and the proton pumping activity of Asp-96 mutants, back to their WT level. We have thus followed the pH dependence of ϕ_r, k_r, and k_d in D96N, in the presence of 10 mM NO_2^-, OCN^- and N_3^-. The relevant parameters obtained by fitting the data to the consecutive kinetic Scheme 2 are shown in Figs. 2 and 3, in comparison with the WT under analogous conditions.

It is evident that the presence of the weak acid anions restores the basic features of the pH effects observed in the WT (Bressler et al., 1999; Balashov et al., 1999): S-shaped curves in the case of ϕ_r, and bell-shaped for k_r and k_d. The data were analyzed by assuming the superposition of a single high-pH titration [$pK_a(1)$], of residue R_1, for all three parameters, and of an additional low-pH titration. In keeping with the model of Bressler et al. (1999), the latter was assigned to a residue, R_2 [$pK_a(2)$], accounting for the S-shaped curve of ϕ_r, and to a different residue, R_3 [$pK_a(3)$], accounting for the bell-shaped curves of both k_r and k_d. Accordingly we used the fitting expression,

$$A(\text{pH}) = B + C_1/(1 + 10^{[\text{pH}-pK_a(1)]}) + C_i/(1 + 10^{[\text{pH}-pKa(i)]}),$$

where C_1 and C_i ($i = 2$ or 3) represent the amplitudes of the titrations of R_1 and R_i ($R_i = R_2$ or R_3), respectively. The related apparent pK_a parameters are shown in Table 1. The principal titration features may be summarized as follows. 1) In D96N, the $pK_a(1)$ titration is markedly downshifted by 1.5–3 units, with respect to the WT. 2) The $pK_a(3)$ titration of k_r and k_d exhibits a much smaller shift of ~0.5 units. Moreover, although the magnitude of the shift in $pK_a(1)$ depends on the specific added anion, the shift in $pK_a(3)$ is similar in all three cases. 3) Due to the small attainable fraction of the ϕ_r titration, imposed by the conversion to the blue form, we could not determine its pK_a value in the

TABLE 1 Estimated pK_a values of the apparent titrations of ϕ_r, k_r, and k_d

System	P ↔ B		ϕ_r		k_r		k_d	
	pK_a*	pK_a	$pK_a(1)$	$pK_a(2)$	$pK_a(1)$	$pK_a(3)$	$pK_a(1)$	$pK_a(3)$
bR[†]		3.5	8.4	4.7	8.1	4.7	7.9	4.5
D96N/N_3^-[‡]	4.5	3.4	6.6	3–4	6.5	3.6	6.5	3.3
D96N/OCN^-[‡]	3.7	3.4	5.8	3–4	5.7	3.5	5.6	3.4
D96N/NO_2^-[‡]	3.3	3.4	5.2	3.6	4.9	3.2	5.0	3.3
bR[§]		3.2	8.1	4.5	8.1	4.3	8.3	4.5
Ac bR[§]		2.9	8.0	3.7	8.3	4.9	8.3	4.9
dI-bR[¶]		6.1	9.6	6.2	9.5	7.2	9.7	7.2
R82Q[‖] (45°C)		–	7.2	–	7.1		6.8	–

The notations (1), (2), and (3) refer to the high pK_a titration of the three parameters (residue R_1), to the low pK_a titration of ϕ_r (residue R_2), and to the low pK_a titration of k_r and k_d (residue R_3). All data, except for R82Q, are at room temperature (21 ± 3°C).

*pKa of weak acid in solution.

[†]dI-bR to which 74 mM NaCl was added.

[‡]10 mM weak acid sodium salt + 40 mM NaCl.

[§]100 mM NaCl.

[¶]Samples of deionized bR (dI-bR) to which 10^{-4} M NaCl were added (Bressler et al., 1999). pK_a values were obtained from the titration data of Bressler et al., (1999), analyzed as described in the text.
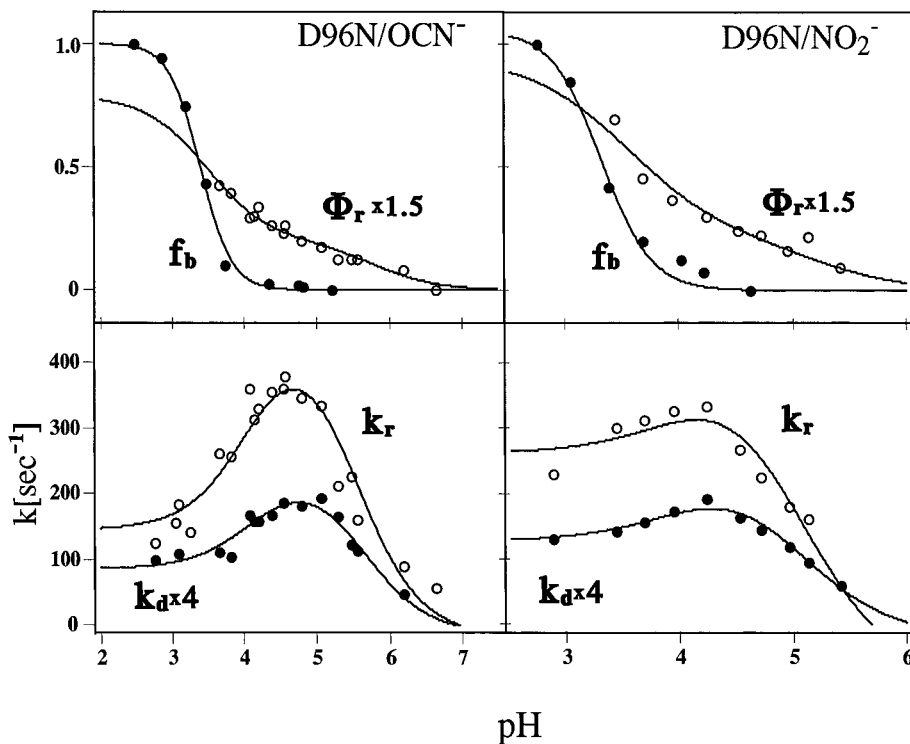
[‖]2 M NaCl.

FIGURE 2 pH effects (experimental points and calculated fits) on ϕ_r, k_r, and k_d in dI-bR to which 74 mM NaCl was added (*left*) and in the D96N mutant in the presence of 10 mM NaN$_3$ and 40 mM NaCl (*right*). f_b is the fraction of blue bR required for correction of the drop in the amount of purple bR at low pH (see details in Bressler et al., 1999).
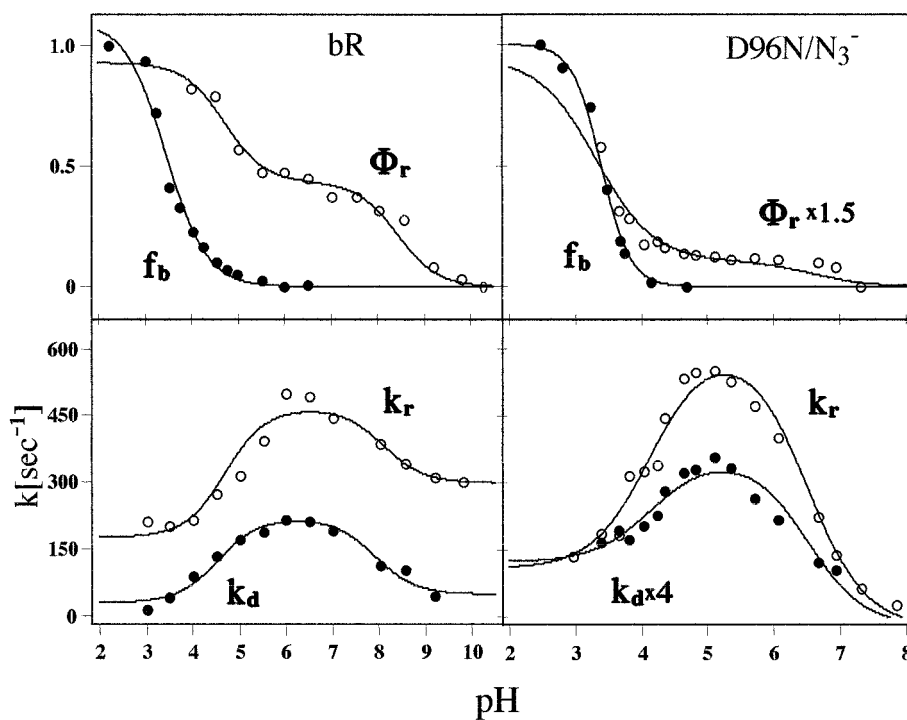
D96N systems. The data can be fitted with pK$_a$(2) values in the range 3–4 (depending on the assumed amplitude of this titration) which appear to be lower than that of the WT (pK$_a$ = 4.7).

These observations suggest that, when incorporated into the protein, the small anions are capable not only of restoring the M$_{412}$ decay kinetics, but also of controlling the pH-dependence of ϕ_r, k_r, and k_d. This interpretation, namely,

FIGURE 3 pH effects in D96N in the presence of NaOCN and NaNO$_2$. Conditions are as for NaN$_3$ in Fig. 2.
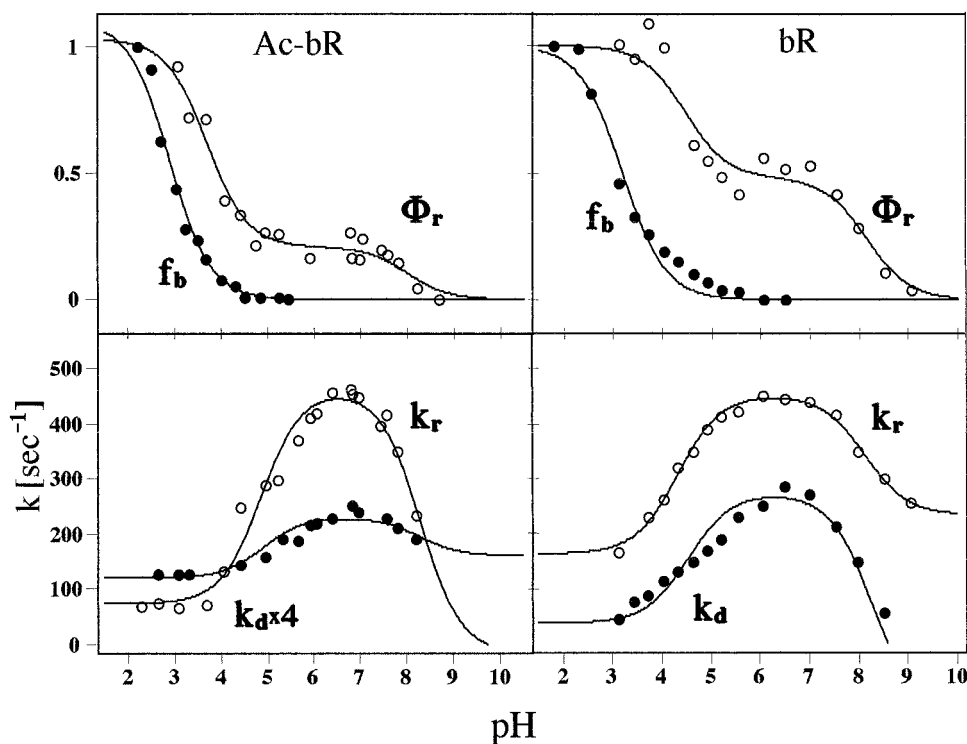
FIGURE 4 Comparison of the pH dependence (experimental points and calculated fits) of the yield and kinetic parameters of O_{630} in bR and in Ac-bR both in the presence of 0.1 M NaCl.

that the anions fully substitute Asp-96 and that the high-pH effect represents their respective titrations, is further supported by the increase in $pK_a(1)$ in the order $NO_2^- < OCN^- < N_3^-$, which closely matches the trend of the corresponding pK_a values of the same anions in solution (see Table 1). Accordingly, when incorporated into bR, the pK_a of the small ions are upshifted by 1–1.5 units with respect to solution. Two mechanisms have been proposed to account for the effect of azide and other small anions on the Schiff base reprotonation rate during the M_{412} decay in the D96N mutant. The first mechanism (Cao et al., 1993a; Titor et al., 1989, 1994) suggests that HN_3 is the active species directly substituting Asp-96 by shuttling protons from the bulk medium to the Schiff base. A different approach (Le Coutre et al., 1995) was based on infrared difference spectra that detected a transient protonation of the azide anion during the photocycle concomitant with reprotonation of M_{412}, rather than the expected deprotonation. It was suggested that azide binds in the extracellular region in the vicinity of Asp-85. Consequently, it accelerates reprotonation of the Schiff base in D96N by restoring the hydrogen-bonded water structure between the Schiff base and Asp-96. Independent of the specific mechanism adopted, the fact that, in the D96N mutant, O_{630} accumulation is restored by the addition of the small anions while $pK_a(1)$ is shifted in accordance with the pK_a of these anions, strongly supports the suggestion of Balashov et al. (1999) that R_1 should be identified with Asp-96. These authors also present a detailed molecular mechanism accounting for the effects of Asp-96 deprotonation at high pH on ϕ_r and k_d. Accordingly, k_d represents the $N_{540} \rightarrow O_{630}$ transition (rather than that of $O_{630} \rightarrow bR_{570}$),

which is controlled by reprotonation of Asp-96. When pH $> pK_a(1)$, the reprotonation of Asp-96 slows down, i.e., k_d decreases, also leading to a drop in ϕ_r.

It is interesting to note that the pK_a of azide at the M_{412} decay stage of the photocycle has been set to 5.3 (Brown and Lanyi, 1996). This value is somewhat higher than that in water (4.5) but lower than that determined in the present work (6.6). This difference is attributed to the fact that the latter value is measured during the lifetime of O_{630}. Accordingly, this would indicate an increase of $\sim$1.5 units during the photocycle interval between M_{412} and O_{630}. As to Asp-96, its pK_a in nonphotocycling bR was determined as 11.4 by Fourier transform infrared spectroscopy (Száraz et al., 1994). During the photocycle, its pK_a was estimated as $\sim$7 at the M_{412} stage (Cao et al., 1993b), and recently, by Fourier transform infrared spectroscopy, as 7.1 (in 1 M KCl) at the stage of N_{540} (Zscherp et al., 1999). The present work indicates that the pK_a of Asp 96 has been increased to $\sim$8 at the stage of O_{630}. This finding should impose restriction on the pK_a of the protonated Schiff base in O_{630}, to avoid reprotonation of Asp-96 by the Schiff base. This can be achieved either by increasing the pK_a of the Schiff base, which was estimated as 8.2 between M_{412} and N_{540} (Brown et al., 1996), or by disturbing the connectivity between Asp-96 and the Schiff base moiety.

Effects of acetylation

Bacteriorhodopsin contains six lysine residues in addition to the retinal binding Lys-216. Five residues are located in the
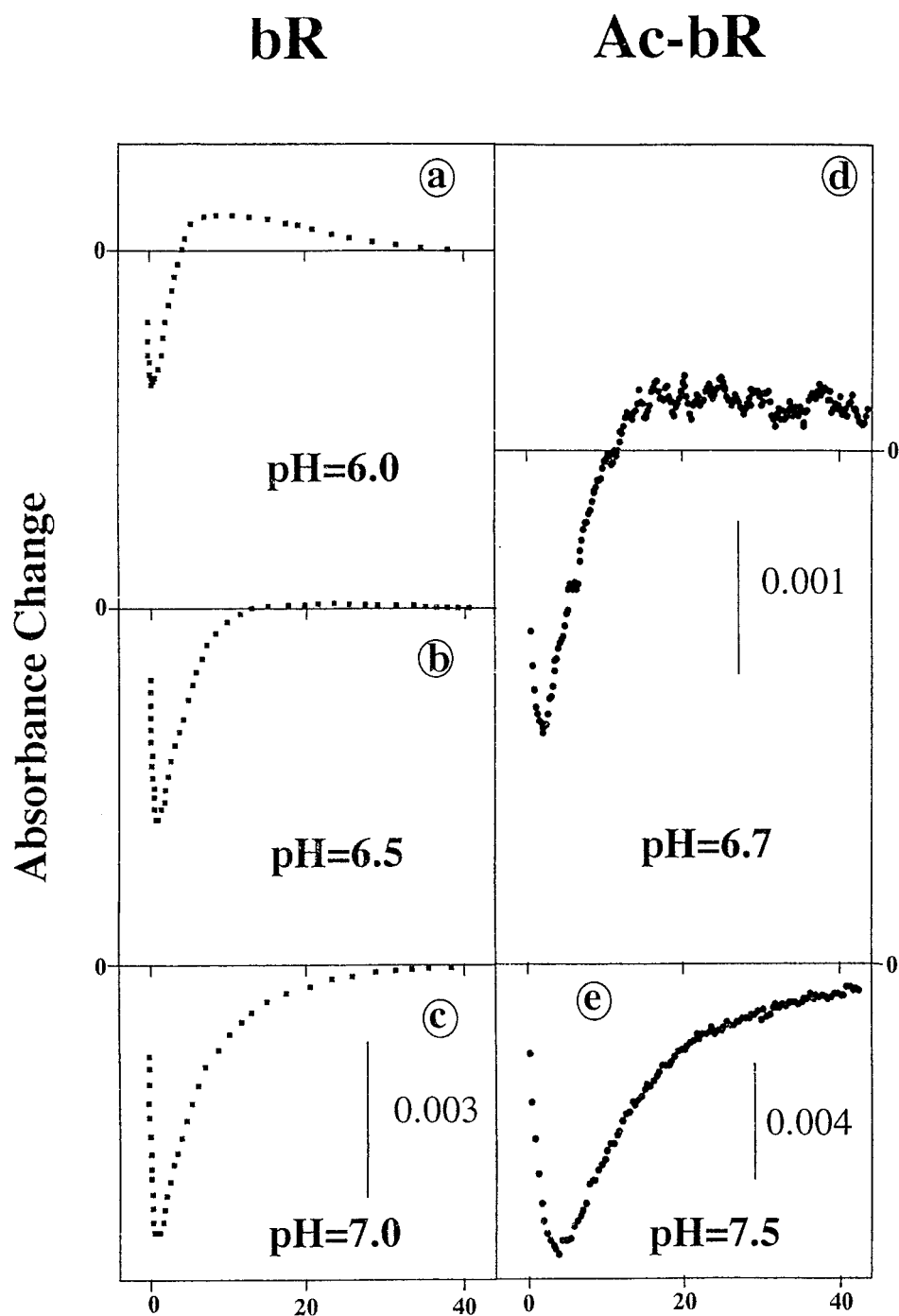
bR   Ac-bR

FIGURE 5 Effects of pH on the proton uptake and release in unmodified bR and Ac-bR (10^{-5} M in 0.1 M NaCl). The former are reproduced from Zimanyi et al., 1992. These of Ac-bR were obtained in the presence of 30 μM pyranine by subtracting transient absorbance traces at 459 nm in the presence of 10^{-3} M buffer, from the corresponding unbuffered suspension at the same pH. Negative and positive deflections reflect proton release and uptake, respectively.

cytoplasmic region, and one (Lys-129) on the extracellular loop connecting the transmembrane helices D and E (Grigorieff et al., 1996). Acetylation of the lysine residues has been found to inhibit proton release during the photocycle, but not proton uptake (Thorgeirsson et al., 1991). This behavior is probably associated with the modification of the pK$_a$ of the proton-releasing moiety (XH), as indicated by the replacement of Lys-129 with histidine, which was shown to increase the pK$_a$ of XH in the M$_{412}$ state, from ~5.8 in the

WT type to ~7.0 (Govindjee et al., 1997). Thus, to examine the suggested identification of either R_2 or R_3 with XH (Balashov et al., 1999), we have investigated the effects of lysine acetylation on pK$_a$(2) and pK$_a$(3), comparing them to the acetylation affects on the pK$_a$ of XH, measured independently.

The pH dependence of ϕ_r, k_r, and k_d in Ac-bR is shown in Fig. 4, in comparison with that of the unmodified pigments under the same conditions (0.1 M NaCl). It is evident
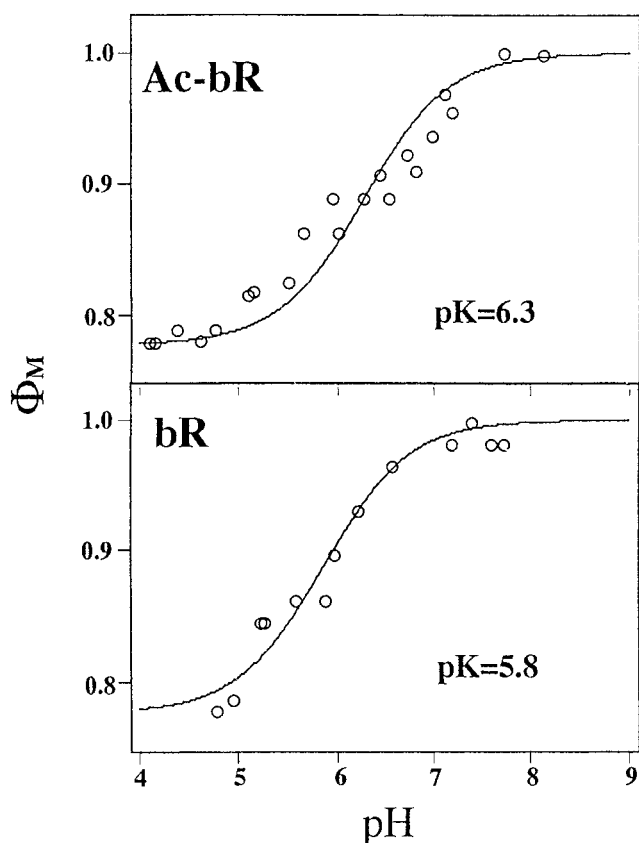
FIGURE 6   Relative amount of the M_{412} intermediate (ϕ_M) as a function of pH. ϕ_M is defined as ΔOD_{412} (pH_i)/ΔOD_{412}(pH7), where ΔOD_{412} represents the absorbance change at 412 nm, 200 μs after excitation, when the $L_{550} \Leftrightarrow M_{412}$ equilibrium is fully established. The relative yield of M_{412} was corrected, on the low pH side, for some conversion into the blue form of bR which does not exhibit an M_{412} intermediate.

that the general pH patterns are maintained in the acetylated system. Analysis of the data presented in Fig. 4 shows that $pK_a(1)$ is essentially unaffected by acetylation. However, the effects on $pK_a(2)$ and $pK_a(3)$ are substantial, and in opposite directions: acetylation increases $pK_a(3)$ by ~0.5 units, whereas $pK_a(2)$ decreases by ~0.8 units.

The effects of lysine acetylation on the pK_a of XH at the photocycle stage in which the proton is released (the $L_{550} \rightarrow M_{412}$ transition) were investigated following proton release and uptake using pyranine as a pH indicator. As shown by Zimanyi et al. (1992) for unmodified bR, the high-pH mechanism in which fast proton release is followed by a slow uptake, is replaced at low pH by a primary (slow) uptake, which is followed by a slower release at the last stage of the photocycle (Fig. 5, *a–c*). The pK_a of this transition ($pK_a \cong 5.8$ in 0.1 M NaCl) was assigned to XH. Analogous experiments for Ac-bR are shown in Fig. 5, *d* and *e*. In these experiments, we have searched for the pH (in the range in which the mechanism turns from high-pH to low pH) which will yield traces analogous to those of unmodified bR. A comparison of Fig. 5, *a*, *b*, and *d* indi-

cates that the XH titration in Ac-bR is upshifted by 0.4–0.6 units. This shift is comparable to that reported for $pK_a(3)$ (but opposite that of $pK_a(2)$), in keeping with the identification of R_3 as XH.

A second, independent, approach for monitoring the effects of acetylation on the pK_a of XH at the photocycle stage in which the proton is released is based on the pH dependence of the $L_{550} \Leftrightarrow M_{412}$ equilibrium. As shown by Zimanyi et al. (1992) and Althaus and Stockburger (1998), this equilibrium exhibits a pK_a of 5.7 (0.15 M NaCl), suggesting that it is controlled by the state of protonation of XH. Experiments with unmodified and Ac-bR, in which the relative amount of M_{412} is monitored as a function of pH, yielded titration curves as shown in Fig. 6. Aceylation up shifts the pK_a of the $L_{550} \Leftrightarrow M_{412}$ equilibrium by 0.6 units, a value that is in keeping with the shifts in $pK_a(XH)$ (Fig. 5) and $pK_a(3)$ (Fig. 4 and Table 1). These observations provide independent support to the mechanism of Althaus and Stockburger (1998) and further establish the identity of R_3 as XH. Thus, $pK_a(XH)$ is ~9.5 in unphotolyzed bR (Balashov et al., 1995, 1996; Richter et al., 1996), 5.8 in M_{412} (Zimanyi et al., 1992; Govindjee et al., 1997), and 4.4 in O_{630}, all in 100–150 mM salt. We note that a low pK_a for the XH moiety in an O intermediate was suggested previously on the basis of studies with artificial pigments (Gat et al., 1997).

The experiments with Ac-bR provide a further example of the distinction between the apparent values of the $pK_a(2)$ and $pK_a(3)$ titrations. Thus, in unmodified bR at high salt (0.1 M NaCl), $pK_a(2)$ and $pK_a(3)$ exhibit a comparable value of ~4.7 (Balashov et al., 1999; Bressler et al., 1999; and Table 1). This analogy breaks down at low salt concentrations (Bressler et al., 1999). The data of Table 1 show that acetylation provides an additional system in which the two pK_a values are substantially separated, and oppositely shifted with respect to the unmodified pigment. These observations may be rationalized by concluding that R_2 and R_3 are different protein moieties, i.e., that R_2 is unrelated to the proton release group. It should be pointed out, however, that, within the framework of a model such as that of Balashov et al. (1999), the maximum fraction of accumulated O_{630} intermediate is a function of k_r and k_d. Accordingly, the apparent pK_a of O_{630} may differ from that of the rate constants, without implying that $R_2 \neq R_3$.

CONCLUSIONS

The yield of the O_{630} intermediate and its apparent rise and decay rate constants are controlled by the state of protonation of several titrable protein residues that have previously been classified (Bressler et al., 1999) as: R_1 (high pH titrations of ϕ_r, k_r, and k_d), R_2 (low pH titration of ϕ_r), and R_3 (low pH titration of k_r and k_d). By studying the pH effects in R82Q and D96N, we have clearly identified R_1 as Asp-96. A comparison of the effects of acetylation on the titrations of R_1, R_2, and R_3 with those of the XH titration is in

keeping with the conclusion (Balashov et al., 1999) that R_3 should be identified with the proton release group. The present conclusions are based on the pH dependence of the apparent rate constants and apparent yield of the O$_{630}$ intermediate, and are thus independent of any specific model of the bR photocycle. A clear identification of R_2, including its possible identity with R_3, will require further studies.

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