Time-Resolved Titrations of the Schiff Base and of the Asp^{85} Residue in Artificial Bacteriorhodopsins[†,‡]

S. Druckmann,[§] M. Ottolenghi,*[,§] I. Rousso,[||] N. Friedman,[||] and M. Sheves*[,||]

Department of Physical Chemistry, The Hebrew University, Jerusalem 91904, Israel, and Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT: Deprotonation/protonation processes involving the retinal Schiff base and the Asp^{85} residue play dominant roles in the light-induced proton pump of bacteriorhodopsin (bR). Although the pK_a values of these two moieties in unphotolyzed bR are well established, the kinetics of the respective titrations in the native pigment are difficult to interpret, primarily due to the extreme (nonphysiological) pK_a values of the two moieties (12.2 ± 0.2 and 2.7, in 0.1 M NaCl, for the Schiff base and for Asp^{85}, respectively). These difficulties are circumvented by applying stopped-flow techniques, time resolving the titrations of several artificial bRs in which the pK_a values of the above two residues are substantially modified: 13-CF_3 bR, pK_a (Schiff base) = 8.2 ± 0.2; 13-demethyl-11,14-epoxy bR, pK_a (Schiff base) = 8.2 ± 0.1 (in 0.1 M NaCl); aromatic bR, pK_a (Asp^{85}) = 5.2 ± 0.1 (in water). The R82Q bR mutant, pK_a (Asp^{85}) ≃ 7.2 was also employed. A major objective was to verify whether the basic relationships of homogeneous kinetics obeyed by elementary acid/base systems in solution (primarily, the possibility to express the equilibrium constant as the ratio of the forward and back rate constants) are also obeyed by the Schiff base and Asp^{85} moieties. We found that this is the case for the Schiff base in the pH range between 7 and 9 but not at lower pH. These observations led to the conclusion that the Schiff base is titrable from the outside medium via a proton channel, which becomes saturated, and thus rate determining, below pH ≅ 7. The observed protonation rate constant in the pH = 7−9 range is $k_a = 6.0 \times 10^7$ M^{-1} s^{-1}, implying a reactivity that is lower by 3 orders of magnitude as compared to the diffusion-controlled rate constant of an elementary acid/base in homogeneous solutions. In the case of Asp^{85}, k_a could not be directly determined. The titration rates observed in the case of pigment IV are, however, consistent with a model in which the Schiff base and Asp^{85} are exposed to the extracellular side via the same proton channel. It is suggested that the rate-determining step in proton translocation via this channel is a transfer between Asp^{85} and the outside, rather than between Asp^{85} and the Schiff base. This conclusion applies independently of whether Asp^{85} is protonated or non-protonated. The results are relevant to basic questions related to the proton pump mechanism in bR, primarily (a) the exposure direction (to the outside or to the inside of the cell) of the Schiff base and of Asp^{85} in unphotolyzed bR and (b) the nature of the still unidentified protein residue (XH) whose proton is translocated to the outside during the bacteriorhodopsin photocycle. We conclude that, in variance with the Schiff base in unphotolyzed bR or with Asp^{85} (in photolyzed or unphotolyzed bR), during the photocycle the XH moiety is highly exposed to the outside medium. More generally, our study bears on the basic problem concerning the relationship between the kinetics of the titration of protein residues and their respective ("thermodynamic") equilibrium constants.

Accumulated evidence indicates that the light-induced proton pump mechanism in bacteriorhodopsin (bR)[1] is directly related to protonation−deprotonation processes of the Schiff base moiety, which links the retinal chromophore to the Lys^{216} protein residue [for recent reviews on bR, see Mathies et al. (1991), Rothschild (1992), Oesterhelt et al. (1992), Ebrey (1993), and Lanyi (1993)]. Illumination of bR induces a cyclic sequential reaction involving a series of spectroscopically distinct intermediates: H, I, J, K, L, M, N, and O. The photon energy, stored during the primary ultrafast events, H → K, is subsequently transformed into a transmembrane proton gradient. Key steps in the proton pump mechanism are the loss of the Schiff base proton, resulting in the M state, and the subsequent reprotonation of the Schiff base from the cytoplasmic side. At least two aspartic residues participate in these processes. Asp^{85}, which becomes protonated during the L → M transition (Braiman et al., 1988; Butt et al., 1989; Stern et al., 1989; Metz et al., 1992), and Asp^{96}, which acts as an intermediate reprotonating agent for the Schiff base during the M → N reaction (Otto et al., 1989; Holz et al., 1989; Gerwert et al., 1989; Butt et al., 1989; Ormos 1991; Sasaki et al., 1992). Of principal interest are also additional roles of the Asp^{85} residue, which is occluded in a hydrophilic region of the protein closer to the extracellular medium, in the vicinity of the Schiff base

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[§] The Hebrew University.

[||] The Weizmann Institute of Science.

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[1] Abbreviations: bR, bacteriorhodopsin; R82Q, bacteriorhodopsin in which arginine 82 has been replaced by glutamine.

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(Henderson et al., 1990). Primarily, the anionic form of Asp^{85}, which determines the purple color of the chromophore, undergoes protonation during an acid-induced transition to the blue form of bacteriorhodopsin, bR_{605} (Mowery et al., 1979; Fisher & Oesterhelt, 1979; Druckmann et al., 1979, 1985; Metz et al., 1992).

The above observations should be considered on the basis of the requirement [see Kalisky et al. (1981)] that the role of light in the H^+ pump mechanism of bR must be associated with either one or a combination of (a) inducing pK_a changes (in the Schiff base and/or in protein residues) and (b) changing the exposure direction (cytoplasmic vs extracellular) of such groups to extra membrane protons. Stimulated work, based on stopped-flow techniques, which not only established the pK_a value of the Schiff base in the dark (Druckmann et al., 1982; Sheves et al., 1986) but also determined the titration rates of the Schiff base (Druckmann et al., 1982) and of its (Asp^{85}) counterion (Druckmann et al., 1979). When applied to vesicles with preferentially oriented purple membranes, stopped-flow acidification data led us to the conclusion that the (Asp^{85}) counterion is accessible to protons from the outside of the cell (Druckmann et al., 1985). Recently, time-resolved experiments, comparing the titrations of the Schiff base in D85N bR and in 13-CF_3 bR, have demonstrated that Asp^{85} is an essential component of the titration channel which connects the Schiff base to the extracellular side (Kataoka et al., 1994).

The kinetic information derivable from time-resolved titrations may prove extremely valuable, especially when compared with the "thermodynamic" pK_a values of the corresponding residues. Thus, in the case of simple equilibria of "free" acid/base groups in homogeneous solutions, the thermodynamic pK_a value equals the ratio of the corresponding (forward and backward) rate constants. However, this relationship is not essentially maintained in nonhomogeneous systems such as proteins. The titration kinetics of protein residues may be complex, reflecting the accessibility of such groups to the extramembrane media. When attempting to challenge these questions in the cases of the Schiff base and Asp^{85} in the native system [see Druckmann et al. (1982)], we encountered several basic difficulties, primarily those imposed by the extreme values of the pK_as of the Schiff base (13.3 in water, 12.2 in 0.1 M NaCl) and of Asp^{85} (2.7 in 0.1 M NaCl). In the case of the Schiff base, this imposes severe protein denaturation effects, and for both residues, it restricts the studies to pH ranges that are not characteristic of the physiological photocycle. Moreover, the wide gap between the two pK_as precludes a comparison between the kinetics of the respective titrations, which may contain valuable information on the accessibility of the two groups to outside protons.

In the present work, we approach the above difficulties by applying time-resolved titrations to bR in which the pK_a value of the Schiff base and/or that of Asp^{85} has been selectively modified either by using artificial pigments based on synthetic chromophores [see Ottolenghi and Sheves (1989) for a review] or by using a genetically modified bR. Our major objectives were (a) to carry out time-resolved titrations of the Schiff base around physiological pH, comparing them to those of a free acid/base in solution; (b) to compare the titration kinetics of the Schiff base and of Asp^{85} in a similar pH range; and (c) to use the resulting data for analyzing the accessibility of both (dark) residues to extramembrane protons, with comparisons to their deprotonation−protonation reactions during the photocycle.

Our data indicate that in unphotolyzed bR, within a restricted pH range (pH ≅ 7−9), the Schiff base moiety behaves similarly to a free acid/base in solution with respect to the relation between the kinetic rate parameters and the equilibrium constant. They also suggest that the titration of the Schiff base and that of the Asp^{85} counterion are carried out via the same proton channel. The results bear on the structure of bR and on its light-induced pump mechanism. Due to the unique property of bR of allowing the spectrophotometric titration of at least two residues (the Schiff base and Asp^{85}), the observations provide rare examples of time-resolved titrations of acid/base groups in a protein. Such titrations allow the consideration of the fundamental problem of the relation between the thermodynamic pK_a value and the rates of acid/base equilibration in protein residues.

MATERIALS AND METHODS

The 13-CF_3 *all-trans*-retinal, 13-demethyl-11,14-epoxyretinal, and aromatic *all-trans*-retinal were synthesized as previously described [Sheves et al. (1986), Brock et al. (1983), and Sheves et al. (1985), respectively]. The corresponding artificial pigments (II−IV) were prepared by incubating the respective aldehyde chromophores at room temperature with the apomembrane (Tokunaga et al., 1978). The R82Q mutant was a generous gift from Profs. R. Needleman and J. Lanyi.

Time-resolved pH-jump experiments, with a time resolution of ~2 ms, were performed using a SHU-PQSF-53 Hi Tech Scientific (Salisbury, U.K.) stopped-flow apparatus. Transient absorbance changes after mixing were processed using a Hi Tech Technical Data Pro software suit. Buffered (10 mM) suspensions of bR membrane sheets were mixed, 1:1, with buffered (100 mM) solutions. Tris buffer (Sigma) was used for the pH range 8−9, phosphate (Merck) was used for pH = 6−8, and phthalate (Fluka) was used for pH ≅ 4.5. Jumps to pH ≅ 2.5 were performed with 0.001−0.01 M HCl solutions. pH values were measured in each suspension prior to [denoted as $(pH)_i$] and after [denoted as $(pH)_f$] mixing. The measured values for the first-order rate constant, for protonation or deprotonation, denoted as k(obs), were obtained by averaging 5−10 pH jumps.

RESULTS

Schiff Base Titration Kinetics in 13-CF_3 bR (Pigment II) and 13-Demethyl-11,14-epoxybR (Pigment III). The general expressions (1 and 2)

$$HB^+ + H_2O \underset{k_a}{\overset{k_d}{\rightleftharpoons}} B + H_3O^+ \quad (1)$$

$$HB^+ + OH^- \underset{k_a'}{\overset{k_d'}{\rightleftharpoons}} B + H_2O \quad (2)$$

which determine the acid/base equilibria of a free acid (HB^+)/base (B) pair in homogeneous aqueous solutions, lead to the elementary equation

$$d[B]/dt = -d[HB^+]/dt = k_d'[HB^+][OH^-] + k_d[HB^+] - k_a[B][H_3O^+] - k_a'[B] \quad (3)$$

which yields the expression

$$k(\text{obs}) = k_d'[\text{OH}-] + k_a' + k_d + k_a[H_3O^+] \quad (4)$$

for the observed rate constant of acid/base equilibration. k_a' and k_d represent the pseudo-first-order rate constants involving water as an acid and a base, respectively. Equation 4 applies to the observed rate constants, in both deprotonation experiments [$k(\text{obs})_d$, corresponding to jumps to higher pH] and protonation experiments [$k(\text{obs})_a$, jumps to lower pH]. The kinetic parameters k_d', k_a', k_d, and k_a are related to the respective thermodynamic equilibrium constants K' and K via eqs 5a, 6a, and 7:

$$k_d'/k_a' = K' \quad (5a)$$

where

$$K' = [B]/[HB^+][OH^-] \quad (5b)$$

and

$$k_d/k_a = K \quad (6a)$$

where

$$K = [B][H_3O^+]/[HB^+] \quad (6b)$$

$$K' = K/([H_3O^+][OH^-]) = K/K_w \quad (7)$$

Equations 4, 5, and 6 apply only when the protonation−deprotonation mechanisms are characterized by single (rate-determining) steps with rate constants k_d, k_a, k_d', and k_a', respectively. In other words, the thermodynamic equilibrium constants, given by eqs 5b and 6b, equal the kinetic constants, eqs 5a and 6a, only when the rate-determining step in each reaction corresponds to those of eq 1 and 2. This is always the case for simple (free) acid/base equilibria in homogeneous solutions but not necessarily for equilibria involving residues in complex, nonhomogeneous structures, such as proteins. Thus, in the case of proteins, access to titrable residues and subsequent protonation−deprotonation may be a complex multistep reaction, which cannot be characterized by eqs 1 and 2 and thus by elementary parameters such as k_d, k_a, k_d', and k_a'. For the sake of clarity, we wish to point out that the term free acid/base equilibrium is used by us with respect to the fulfillment of eqs 4, 5, and 6. This does not essentially imply that in proteins k_d and k_a are the same as in the case of an elementary acid in solution. Thus, in the case of a protein equilibrium, k_a may be smaller that the diffusion-controlled rate constant observed for an elementary acid in homogeneous solutions while still conforming to eqs 4, 5, and 6.

We have previously attempted to challenge this question by carrying out fast pH jumps involving protonation or deprotonation of the Schiff base in native bR (Druckmann et al., 1982) but were faced with two major difficulties: (a) Due to the high pK_a and thus the high pH range involved in the native system, both equilibria, eqs 1 and 2, must be considered, requiring the fit of the five parameters k_a, k_d, k_a', k_d', and K. (b) The titrations from physiological conditions (i.e. pH = 6−7) to high pH (pH >12) and vice versa involve substantial structural changes in the protein. In other words, due to the titration of other protein residues, and in variance with simple (free) acid/base residues, a protein species such as HB^+ (in the present case, the protonated Schiff base) at pH ≅ 7 may not be identical to the same species at pH 12.5 with respect to its microenvironment and H^+ channels. This creates additional difficulties in the application of eqs 1 and 2.

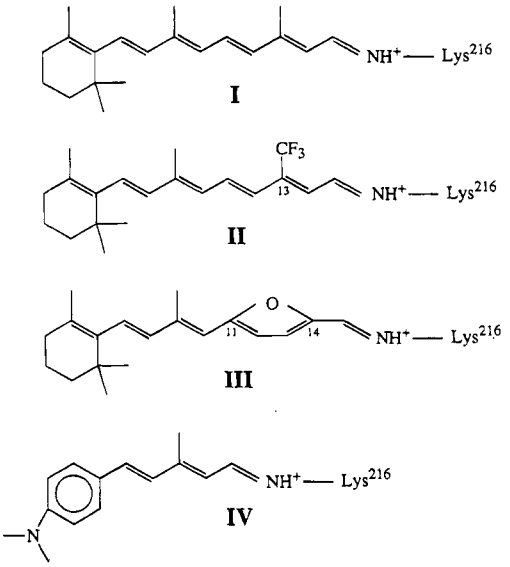

FIGURE 1: Chromophores of native bR and of artificial bRs studied in the present work. (I) Native bR. (II) 13-CF_3 bR. (III) 13-Demethyl-11,14-epoxybR. (IV) Aromatic bR.

In the present work, we circumvent these difficulties by using two artificial pigments, 13-CF_3 bR (II) and 13-demethyl-11,14 epoxy bR (III) (Figure 1), in which the pK_a of the protonated Schiff base is drastically reduced, to pK_a = 8.2 ± 0.2 (Sheves et al., 1986) and to pK_a = 8.2 ± 0.1 (Rousso et al., 1995), respectively. Note that in pigment III Asp^{85} undergoes a drastic increase in pK_a, so that it maintains its protonated form over the whole pH 1−9 range (Rousso et al., 1995). The reduction of the Schiff base pK_a in these pigments allows us to work closer to physiological conditions, not only avoiding high-pH structural changes and/or denaturation effects but also allowing for neglecting equilibration via eq 2. Thus, if the protein Schiff base residue behaves like a free acid/base couple, eq 4 is replaced by the simpler form

$$k(\text{obs}) = k_d + k_a[H_3O^+] \quad (8)$$

A characteristic jump from the non-protonated form (λ_{max} = 420 nm) to the protonated form (λ_{max} = 630 nm) in the case of the 13-CF_3 pigment is shown in Figure 2b. The experiment was repeated for a series of final pH values in the 0.93 < $(pH)_f$ < 7.50 range. Similarly, opposite jumps from low to high pH were performed (e.g., Figure 2a). The observed rate constants are presented in Table 1.

Several major conclusions may be derived from the data of Table 1. First, as shown in Figure 3a, a linear relationship between $k(\text{obs})$ and $[H^+]$ is observed in the range between $(pH)_f \cong 9$ and 7. It is important to note that both $k(\text{obs})_d$ and $k(\text{obs})_a$ fit the same curve, in keeping with eq 8. Moreover, the values of the rate constants derived from Figure 3a, namely, $k_d = 0.9\ s^{-1}$ and $k_a = 6.0 \times 10^7\ M^{-1}\ s^{-1}$ (50 mM buffer), yield a rate constant ratio $k_d/k_a = 1.5 \times 10^{-8}$ ($pK_a = 7.8 \pm 0.5$), which fairly fits the observed value of the thermodynamic equilibrium constant of the Schiff base titration, namely, $pK_a = 8.2 \pm 0.2$ in 0.1 M NaCl. We therefore conclude that, within the range 7 < $(pH)_f$ < 9, the Schiff base of 13-CF_3 bR behaves like a free acid/base with

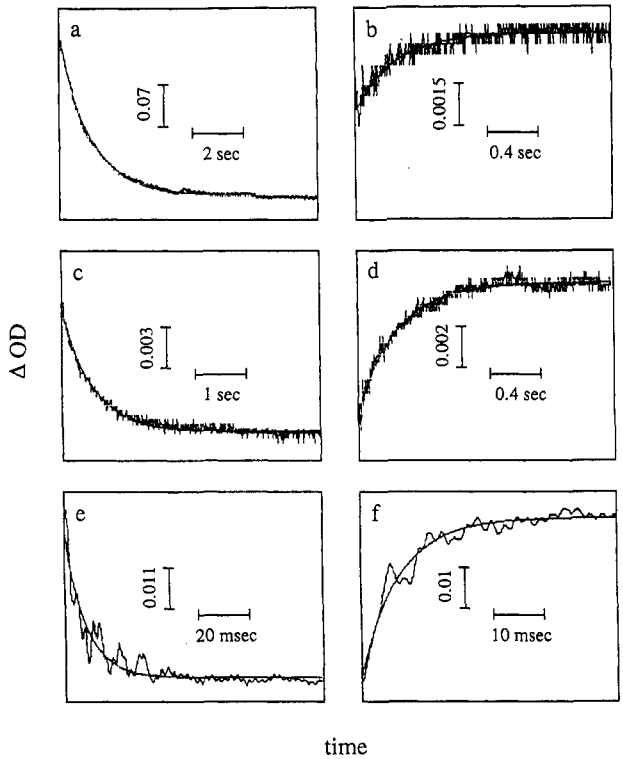

FIGURE 2: Characteristic traces of fast pH jumps of artificial bacteriorhodopsins measured by the stopped-flow technique (λ values denote measuring wavelengths). (a) Deprotonation of the SB of 13-CF_3 bR (λ = 630 nm). $(pH)_i$ = 4.90; $(pH)_f$ = 8.99. (b) Protonation of the SB of 13CF_3bR (λ = 630 nm). $(pH)_i$ = 8.60; $(pH)_f$ = 7.50. (c) Deprotonation of the SB of 13-demethyl-11,14-epoxybR (λ = 570 nm). $(pH)_i$ = 6.08; $(pH)_f$ = 8.66. (d) Protonation of the SB of 13-demethyl-11,14-epoxybR (λ = 570 nm). $(pH)_i$ = 9.06; $(pH)_f$ = 7.09. (e) Deprotonation of Asp^{85} of aromatic bR (λ = 630 nm). $(pH)_i$ = 4.50; $(pH)_f$ = 6.24. (f) Protonation of Asp^{85} of aromatic bR (λ = 630 nm). $(pH)_i$ = 7.00; $(pH)_f$ = 4.55.

respect to the simultaneous fulfillment of both eq 8 and eq 6. However, at variance with homogeneous solutions where k_a is diffusion controlled, i.e., $k_a = 10^{10}-10^{11}$ M^{-1} s^{-1}, the value observed in the case of 13-CF_3 bR is smaller by more than 3 orders of magnitude. Thus, in this respect, the titration of the Schiff base differs from that of a fully free acid in solution. It is important to note that eq 8 is fairly maintained for all $(pH)_f$ values in the range between $(pH)_f \cong 7$ and 9, even when $(pH)_i$ varies between 4.5 and 8.6. This implies that the initial pH [within the 8.0 < pH < 8.9 range for protonation experiments and within the 4.5 < pH < 6.8 range for deprotonation experiments] does not markedly affect the titration. This means that no structural changes affecting the titration are induced by the above variations in $(pH)_i$ or, alternatively, that if present, such changes are cancelled by the pH jump from $(pH)_i$ to $(pH)_f$ on a time scale which is faster than the titration of the Schiff base.

It is evident from the data of Table 1 that the free acid/base behavior of the Schiff base, namely, the fulfillment of both eqs 8 and 6, is limited to the range $7 < (pH)_f < 9$. Below $(pH)_f \cong 7$ (down to pH $\cong$ 1) the pH dependence of $k(obs)_a$ becomes nonlinear and approximately 2 orders of magnitude weaker than the linear one in Figure 3a. This implies a drastic deviation from eq 8 as maintained in the pH 7−9 interval.

Experiments similar to the above were also carried out with 13-demthyl-11,14-epoxybR (III, depicted in Figure 1). The corresponding k(obs) data (given in Table 1 and Figure 3b) show a general trend similar to that of 13-CF_3 bR (II): A relatively strong dependence of k(obs) [for both $k(obs)_d$ and $k(obs)_a$] on $[H^+]$ within a relatively narrow range [$7.6 < (pH)_f < 8.7$ as shown in Figure 3b] and a much weaker dependence on $[H^+]$ at lower pH values (data in Table I, not plotted). Unfortunately, due to the relatively large experimental error in the values of k(obs), a quantitative test of the data in terms of eq 8 could not be carried out. However, as shown in Figure 3b, the linear plot high-pH range is not inconsistent with a line characterized by the parameters $k_a = 6.6 \times 10^7$ M^{-1} s^{-1} and $k_d = 1.1$ s^{-1}, which are close to those obtained for pigment II, implying a kinetic pK_a value of ~8, which agrees with the thermodynamic value $pK_a = 8.2 \pm 0.1$ (in 0.1 M NaCl) (Rousso et al., 1995). Since the scattering of our experimental points in this range implies an uncertainty of about 1 unit in the kinetic pK_a value, we are limited to the general conclusion that the behavior of pigment III qualitatively resembles that of pigment II with respect of the fulfillment of eqs 8 and 6.

Titration of Asp^{85} in Aromatic bR and in R82Q bR. The aromatic-bR pigment (IV), characterized by a shorter polyene chain and by the replacement of the cyclohexene retinal ring by an aromatic ring, exhibits a protonated Schiff base pK_a (12.0 ± 0.2 in 0.1 M NaCl) which is close to that of native bR (Rousso et al., 1995). However, the pK_a of the Asp^{85} residue as monitored by the characteristic red shift induced at low pH is increased by approximately 2 units, to $pK_a \cong 5.2 \pm 0.1$. This value reduces the gap between the pK_a of Asp^{85} and that of the Schiff bases of 13-CF_3 bR (II) and pigment (III), to about 3 units, as compared to more than 10 units in the case of the same two moieties in the native system. This allows us to carry out comparative time-resolved titrations of the two groups in relatively close pH ranges within the physiological range, avoiding the complications imposed by jumps to extreme pH values, i.e., below ~3 and above ~12.

The k(obs) parameters recorded for pigment IV over a range of $(pH)_i$ and $(pH)_f$ values are given in Table 2. Nearly overlapping pH jumps, which titrate the Schiff base in pigment II and Asp^{85} in pigment IV [with similar $(pH)_i$ and $(pH)_f$ values], are specifically tagged in Tables 1 and 2. A more extensive range of overlapping pH values for the two titrations was precluded by the observation that above pH $\cong$ 7.2 pigment IV begins to undergo a transition to a still uncharacterized blue-shifted modification (λ_{max} = 496 nm; pK_a = 7.8). This imposes an upper limit on the pH in experiments with this pigment. As for pigment II, its relatively high pK_a limited $(pH)_i$ in the protonation experiments to $(pH)_i \geq 8$. It is evident that the titration kinetics of Asp^{85} in pigment IV are much faster than the titration of the Schiff bases of the two artificial pigments, (II) and (III). At variance with the latter titration rates, which are pH dependent, an apparently pH-independent value is observed in the case of the titration of Asp^{85} in the aromatic chromophore for both $k(obs)_a$ and $k(obs)_d$ over the $4.5 < (pH)_f < 7.2$ range.

Experiments determining $k(obs)_d$ and $k(obs)_a$ at single $[H^+]_i$ and $[H^+]_f$ values were also carried out with the R82Q mutant of bR, which, similarly to the aromatic pigment (IV), exhibits a relatively high value for the acid dissociation constant of Asp^{85} ($pK_a \cong 7$) while maintaining a Schiff base pK_a analogous to that of the wild type (Stern et al., 1989;

Table 1: Observed Rate Constants for the Titration of the Schiff Base in the Artificial Pigments 13-CF_3bR and 13-Demethyl-11,14-epoxy-bR[a]

13-CF_3bR (II)			13-demethyl-11,14-epoxy-bR(III)			native bR (I)		
$(pH)_i$	$(pH)_f$	k(obs) (s^{-1})	$(pH)_i$	$(pH)_f$	k(obs) (s^{-1})	$(pH)_i$	$(pH)_f$	(k(obs) (s^{-1})
		[$k(obs)_d$]			[$k(obs)_d$]			[$k(obs)_d$]
4.90	8.99	0.8	6.08	8.66	1.3	6.50	12.48	0.04
6.77	8.19	1.7	6.48	8.65	0.8	6.50	12.67	0.11
4.47	7.56	2.6	6.08	8.42	1.8	6.50	12.82	0.26
4.47	7.35	3.3[b]	6.08	8.30	2.0			
4.47	7.14	5.0[b]						
		[$k(obs)_a$]			[$k(obs)_a$]			[$k(obs)_a$]
8.60	7.50	4.0	8.74	7.98	1.2	12.67	10.00	47
8.18	7.28	3.5	8.74	7.94	1.8	12.67	9.20	210
8.90	7.12	5.8	8.22	7.64	2.8	12.67	7.00	>1000
7.97	7.03	4.7	8.40	7.50	2.7			
8.18	6.77	12.0	8.74	7.36	2.4			
7.97	6.55	12.2	8.40	7.19	2.2			
7.97	4.72	19.2[c]	9.06	7.09	2.9			
8.60	2.02	37.0	8.22	6.79	3.7			
8.60	0.93	42.0	8.74	6.33	3.6			
			8.74	6.21	4.6			
			8.22	6.04	7.0			

[a] $k(obs)_d$ (upper values) and $k(obs)_a$ (lower values) represent the observed deprotonation and protonation rate constants, respectively. $(pH)_i$ and $(pH)_f$ represent the initial and final pH values, respectively. Data for native bR are reproduced from Druckmann et al. (1982). [b,c] Denote experiments that will be compared with analogous titrations of Asp^{85} (see subsequent discussion and footnote in Table 2).

Subramaniam et al., 1990; Drachev et al., 1992; Balashov et al., 1993; Brown et al., 1993). The data are presented in Table 2.

DISCUSSION

Schiff Base Titration Rates in Low-pK_a bR Mutants: Comparison with Native bR. We have previously shown (Sheves et al., 1986) that in 13-CF_3 bR (pigment II) the shift in the pK_a of the Schiff base with respect to the native pigment (~5 pK_a units) is similar to the shift between the pK_as of the free Schiff bases of the corresponding chromophores in solution. This provides conclusive evidence that in the artificial pigment the pK_a shift is induced intrinsically by the electron-withdrawing effect of the CF_3 substituent. At variance with the effect of CF_3 substitution, the reduction of the pK_a of the Schiff base in the case of 13-demethyl-11,14-epoxy-bR (pigment III) is due to chromophore protein interactions rather than to intrinsic properties of the chromophore (Rousso et al., 1995). It appears that, independently of the cause which is responsible for inducing the pK_a shift, the same major conclusion is derived from the pH jumps of these two artifical pigments, namely, that they both comply with our definition of free acid/base pairs. This conclusion is clearly evident in the case of pigment II for which the linear relationship in eq 8 is maintained, yielding kinetic parameters k_a and k_d which closely fit the thermodynamic value of the respective equilibrium constants. In other words, protonation and deprotonation from the external medium are controlled by two rate-determining steps, with protonation being proportional to $[H^+]$. This feature is not observed in the case of native bR, for which $k(obs)_a$ qualitatively obeys eq 8, but no fits with $k(obs)_d$ and the pK_a value of 13.3 (in water), eqs 5−7, were obtained (Druckmann et al., 1982). This discrepancy is most probably due to the complicating effects of the additional high-pH titration path (eq 2) involving OH^- ions, as well as to protein structural changes induced by the high pH required for the titration in the native system. Both are apparently absent in the titrations of the two low-pK_a artificial pigments, which are carried out at much lower pH values.

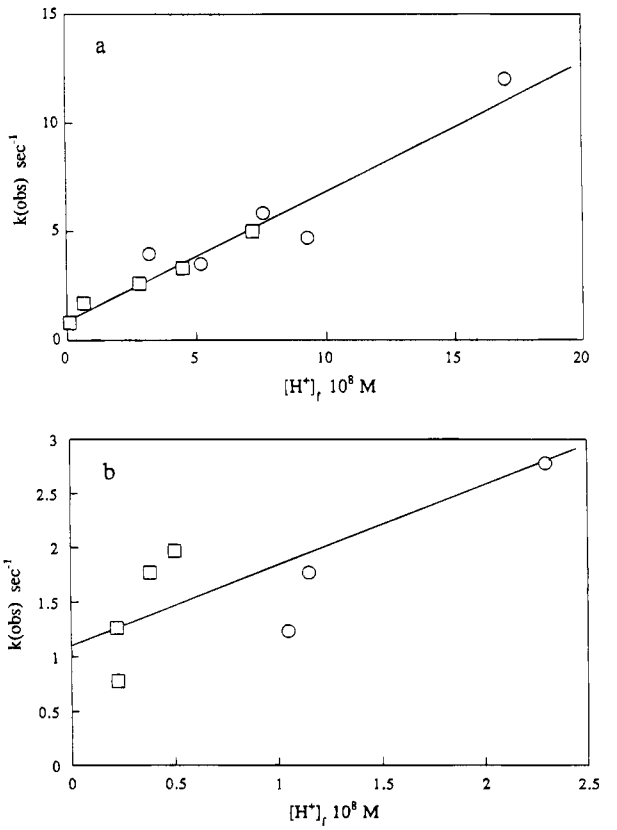

FIGURE 3: (a) $[H^+]$ dependence (in the high-pH range) of the observed rate constants for the time-resolved titration of the Schiff base of 13-CF_3 bR. (Points correspond to those reported in Table 1 for $(pH)_f$ values between 6.77 and 8.99) (○) $k(obs)_a$ (protonation experiments); (□) $k(obs)_d$ (deprotonation experiments). (b) $[H^+]$ dependence of k(obs) in the time-resolved titrations of 13-demethyl-11,14-epoxybR (data presented in Table 1). (○) $k(obs)_a$ (protonation experiments); (□) $k(obs)_d$ (deprotonation experiments). The solid line is arbitrary drawn so as to fit eq 8 with the parameters k_a = 6.6×10^7 M^{-1} s^{-1} and k_d = 1.1 s^{-1}.

When analyzing the Schiff base protonation rates of native bR for jumps from $(pH)_i \simeq 12.7$ to $(pH)_f = 7-10$, we obtained an essentially diffusion-controlled value for k_a (k_a

Table 2: Observed Rate Constants for the Titration of Asp^{85} in Aromatic bR and R82Q bR[a]

aromatic bR (IV)			R82Q bR (V)			native bR (I)		
$(pH)_i$	$(pH)_f$	$k(obs)$ (s^{-1})	$(pH)_i$	$(pH)_f$	$k(obs)$ (s^{-1})	$(pH)_i$	$(pH)_f$	$k(obs)$ (s^{-1})
		$[k(obs)_d]$						
4.50	6.24	120	7.10	9.00	660 $[k(obs)_d]$	2.50	7.03	184 $[k(obs)_d]$
4.50	6.91	130[b]						
2.98	7.05	140						
4.50	7.25	130[b]						
7.00	4.55	120[c] $[k(obs)_a]$	8.10	7.02	30 $[k(obs)_a]$	7.00	2.10−2.70	30 $[k(obs)_a]$

[a] A comparative value for $k(obs)_a$ of native bR is reported from Druckmann et al. (1979). [b] Denotes $k(obs)_d$ values obtained under $(pH)_i$ and $(pH)_f$ conditions, which match those similarly tagged in Table 1 in the case of the titration of the Schiff base of pigment II. [c] Denotes a $k(obs)_a$ value obtained for a $(pH)_f$ value similar to that analogously tagged in Table 1 for the Schiff base titration of pigment II.

$\simeq 10^{11}$ M^{-1} s^{-1}), which is higher by 4 orders of magnitude than that of the present low-pK_a artificial pigments. We attribute this difference to the role played by the *initial* (high) pH in the Schiff base protonation experiments in the native system. At pH $\gtrsim$ 12.5, a partially reversible denaturation of the protein takes place [see Druckmann et al. (1982)], which exposes the Schiff base binding site to the external medium, leading to very high protonation rate constants. The relatively low value of k_a ($\simeq 6 \times 10^7$ M^{-1} s^{-1}) observed for the two artificial pigments, II and III, implies slower diffusion rates to the binding site or a smaller cross section for the reaction between the Schiff base and H^+, though still maintaining $k_a[H^+]$ as the rate-determining expression of the protonation reaction.

As discussed above, the basic feature of the Schiff base titration, namely, the behavior as a free acid/base system (although within a limited pH range), is observed for both artificial pigments, with similar k_a and k_d parameters. It should be noted that the factors inducing the reduction in the respective pK_as are different, while the basic titration rate features are similar for the two chromophores. This implies that the protonation and deprotonation rates and paths are basically governed only by the pH and (possibly) by the pK_a of the titrable Schiff base residue. The question arises, however, concerning the loss of the strong linear dependency of $k(obs)$ on $[H^+]_f$ at relatively low $(pH)_f$ values. Although differing in detail, this feature is qualitatively common to both 13-CF_3 bR (pigment II) and pigment III. It implies that when $[H^+]_f$ reaches sufficiently high values, the $[H^+]_f$ dependency of $k(obs)$ is markedly reduced, meaning that $k_a[H^+]$ is no longer the rate-determining expression. The effect may be qualitatively rationalized by implying the existence of a proton channel, from the Schiff base to the extramembrane medium, which becomes "saturated" beyond a certain $[H^+]$ value. In other words, at low $[H^+]$ the number of protons passing through the channel per unit time is proportional to $[H^+]$. However, when the channel reaches saturation at higher $[H^+]$, the rate of proton transfer through the channel becomes essentially independent of $[H^+]$. It should be pointed out that this (saturation) mechanism may hold without requiring a (low) pH-induced structural change of the channel. Obviously, both effects may be operative. For example, it is possible that the weak residual dependence on $[H^+]$ at low pH observed for both artificial pigments is due to structural changes in the channel induced by the titration of protein residues.

An additional point of significant relevance is the effect of the state of protonation of Asp^{85} on the rate of protonation and deprotonation of the Schiff base. In the preceding paper, we have presented substantial evidence suggesting that under the pH conditions of our experiments (pH < 9) the Asp^{85} residue in 13-demethyl-11,14-epoxy-bR is protonated. Should this suggestion be finally confirmed by direct independent evidence (for example, by FTIR measurements), it will be implied that the two pigments, II and III, exhibit similar titration patterns for the Schiff base despite the different state of protonation of Asp^{85}. In other words, the state of protonation of Asp^{85} does not seem to affect the connectivity of the Schiff base with extramembrane protons. The implications of this conclusion will be further considered in the subsequent discussion. It is relevant, however, to recall the observation (Kataoka et al., 1994) that the replacement of Asp^{85} by asparagine slows down the rate of proton transfer from and to the Schiff base [$k(obs) \sim 0.02$ s^{-1} at $(pH)_f = 8$] by a factor of 50 with respect to pigment II and also, as shown in the present work, with respect to pigment III. Thus, the complete replacement of the 85-aspartate residue by an uncharged group appears to produce additional effects beyond those induced by protonation.

Titration of Asp^{85}. In the case of the (Asp^{85}) Schiff base counterion of native bR, we have previously reported protonation experiments corresponding to pH jumps from $(pH)_i \simeq 7$ to $(pH)_f \simeq 2.1-2.7$ (Druckmann et al., 1979). The corresponding $k(obs)_a$ values, along with a value for $k(obs)_d$ obtained in the present work, are given in Table 2. Due to the limitations imposed by the relatively low pK_a of Asp^{85} in native bR, similar experiments could not be carried out closer to the higher $(pH)_f$ range (7 < pH < 9) where the Schiff base of 13-CF_3 bR (pigment II) as well as that of pigment III were titrated. As shown in the present work, the pH range in which the titration takes place is of primary importance in determining the titration kinetics.

In order to circumvent this difficulty, we have carried out experiments comparing the Schiff base titrations in 13-CF_3 bR and 13-demethyl-11,14-epoxy-bR with those of Asp^{85} in aromatic bR (IV) and R82Q bR, which both take place close to neutral pH. The combination of an aromatic core and the short polyene chain in pigment IV probably induces a spatial alteration in the protonated Schiff base/Asp^{85} structure, thereby decreasing the stabilization of the Asp^{85} negative charge by bound water. This effect is presumed to induce the higher pK_a for Asp^{85} in this pigment (Rousso et al., 1995). In R82Q bR, the pK_a of Asp^{85} is increased (Balashov et al., 1993), probably due to elimination of the positive charge of Arg^{82}, which might be located in the vicinity of Asp^{85} (Henderson et al., 1990).

As shown in Table 2, the titration of Asp^{85} in the aromatic pigment IV shows a $k(obs)_d$ value of $\sim$130 s^{-1}, which is

independent of $(pH)_f$ and $(pH)_i$ in the $6.2 < (pH)_f < 7.2$ and $2.9 < (pH)_i < 4.5$ ranges to which our experiments were restricted. In terms of eq 8 the apparent $(pH)_f$ independency of $k(obs)_d$ may be due to a negligible contribution of the term $k_a[H^+]$, relatively to k_d. Alternatively, it is possible that eq 8 is not at all applicable.

A direct discrimination between these alternatives is precluded by the relatively narrow $(pH)_f$ range to which our present deprotonation experiments are inherently restricted. It is tempting, however, to postulate (Druckmann et al., 1985; Kataoka et al., 1994) that Asp^{85} and the Schiff base are both exposed to the extracellular side, sharing the same proton channel. In such a case, one may assume that the two titrations are characterized by the same k_a value ($\sim 6 \times 10^7$ M^{-1} s^{-1}). Using this value in the case of the Asp^{85} titration above pH $\cong$ 7 shows that $k_a[H^+] \ll k_{obs}$, which will account for the apparent pH independence of $k(obs)_d$. [The failure to observe some increase in k(obs) between pH $\cong$ 7 and 4.5 may be due to entrance into the range in which the pH dependency is considerably weaker.] Also in keeping with the joint channel assumption is the ratio $k_d/k_a = 130/6.0 \times 10^7 = 2.2 \times 10^{-6}$, implying $pK_a = -\log (k_d/k_a) \cong 5.7$, which is close to the thermodynamic pK_a value (5.2) of this pigment.

According to this interpretation, the k_d value for Asp^{85} is on the order of 10^2 s^{-1}, as compared to ~ 1 s^{-1} in the case of Schiff base titration of the Schiff base of pigments II and III. This may be consistent with a mechanism in which the rate-determining step in the apparent deprotonation of the Schiff base is the very deprotonation reaction. However, in the case of Asp^{85}, which is an acid stronger by 2−3 pK_a units, the properties of the channel become rate determining, imposing the upper limit of $\sim 10^2$ s^{-1}. These conclusions could, in principle, be verified by examining the rates of protonation of Asp^{85} in pigment IV at pH values well below pH $\cong$ 6, thus increasing the weight of the $k_a[H^+]$ term [see Table 2, reporting a $k(obs)_a$ value of 120 s^{-1} for a jump to $(pH)_f = 4.55$]. The problem with this kind of test is our conclusion that eq 8 [i.e., $k(obs) = 0.9 + 6.0 \times 10^7[H^+]$) does not apply at low pH. This practically implies that the effective k_a value below pH $\simeq$ 7 is about 2 orders of magnitude smaller than the high pH value 6.0×10^7 M^{-1} s^{-1}, thus cancelling the increase in $[H^+]$. It is interesting to note that the differences between the titration rates of the Schiff base and of Asp^{85}, as seen in the physiological pH range, are not observed at very low pH. Thus, as shown in Tables 1 and 2, the values of $k(obs)_a$ for jumps to pH $\cong$ 2 appear to be essentially the same for the titration of Asp^{85} in the native pigment [$k(obs)_a = 30$ s^{-1}] and for the titration of the Schiff base in 13-CF_3 bR (II) [$k(obs)_a = 37$ s^{-1}]. This behavior is in keeping with a joint rate-determining step in the titration of the two residues also at low pH.

In conclusion, although still awaiting verification by the direct determination of k_a for Asp^{85}, our data are in keeping with the possibility that the Schiff base and Asp^{85} are both exposed to the extracellular side, via the same proton channel. Since the Schiff base titration is qualitatively the same for pigments II and III, it appears to be independent of the state of protonation of Asp^{85}. In other words, the rate-determining step appears to be H^+ transfer via the channel connecting Asp^{85} and the extracellular side, rather than transfer between Asp^{85} (or $Asp^{85}H$) and the Schiff base. When Asp^{85} is completely replaced by a neutral residue, such as asparagine, $k(obs)_d$ decreases by more than an order of magnitude (Kataoka et al., 1994). This may imply a structural perturbation (not observed upon simple protonation), which completely disrupts the channel.

We finally note that the above arguments and conclusions apply to the artificial pigment IV, but not to the R82Q mutant. It is evident that the two k(obs) values available for this pigment (V), reported in Table 2, are inconsistent with eq 8; i.e., k(obs) decreases while $[H^+]$ increases. It appears that the replacement of Arg^{82} induces changes in the binding site and in the related proton channels that preclude any simple comparison with either native or artificial bacteriorhodopsins.

On the Rate of Hydrogen/Deuterium Exchange by the Schiff Base. It is interesting to compare the rates of protonation and deprotonation of the Schiff base, presented in the current work, with those observed for hydrogen/deuterium (H/D) exchange rates of the same moiety. (H/D exchange rates for the Asp^{85} residue are still unavailable.) It has been shown (Doukas et al., 1981) that the time scale of H/D exchange of the Schiff base in bR is shorter than several milliseconds. This rate is considerably faster than that predicted by mechanisms based on base (H_2O or OH^-)-catalyzed deprotonation followed by reprotonation. Such mechanisms were therefore excluded, leading to an alternative exchange mechanism in which a water molecule interacts directly with the Schiff base deuteron in a concerted exchange step. The inapplicability of the base-catalyzed mechanism is further supported by the actual protonation/deprotonation rates measured for the Schiff base in the present work, which are slower by several orders of magnitude than that of H/D exchange. The latter has been recently resolved and shown to occur with a half-life of 1.3 ms (Deng et al., 1994), under pH conditions analogous to those in which we measured protonation/deprotonation for pigments II and III.

The concerted exchange mechanism, involving a water molecule interacting with the Schiff base, is based on H/D exchange between the external aqueous medium and the exchanging H_2O molecule, followed by the exchange between the latter and the Schiff base. Deng et al. (1994) have proposed that the exchanging water molecule is structurally positioned in the retinal binding site, as part of an arrangement, as discussed in the preceding paper (Rousso et al., 1995). Since the rate-determining step in such a sequential exchange mechanism has not yet been established, it is impossible to interpret the exact meaning of the 1.3 ms exchange lifetime and thus to compare it with the presently reported protonation/deprotonation states. It will be interesting, however, to establish whether the perturbation of the binding site sterically induced by the synthetic chromophores described in this and the preceding paper (Rousso et al., 1995) (which results in a reduced Schiff base pK_a) will also affect the rates of H/D exchange.

Conclusions: Relevance to the Structure of bR and to Its Photocycle Mechanism. Using artificial bacteriorhodopsins with reduced protonated Schiff base pK_as, we have shown that the basic relationships of homogeneous kinetics, eqs 6 and 8, apply to the titration of this bR moiety, although within a restricted (physiological) $7 < pH < 9$ range. Analogous pH jumps with an artificial bacteriorhodopsin with a modified pK_a of Asp^{85} shows that this might also be the case for the latter residue, which is situated in the vicinity of the Schiff base. These observations may be rationalized in terms of a

mechanism in which Asp^{85} is an essential part of the channel, which allows proton exchange between the Schiff base and the extramembrane medium. Moreover, we suggest that the state of protonation of Asp^{85} does not affect the rate of proton translocation along the channel. The latter is attributed to proton translocation between Asp^{85} and the extracellular side rather than between Asp^{85} and the Schiff base. The conclusion that Asp^{85} is an essential component of the channel to the Schiff base is in keeping with recent observations (Kataoka et al., 1994) showing that in a bR mutant in which Asp^{85} is replaced by asparagine the rate of Schiff base deprotonation is markedly reduced. On the basis of this observation and the effects of added azide on the protonation rates in the cases of D85N/D96N and D85N/D96A, it was argued that in the absence of Asp^{85}, which connects the Schiff base to the outside of the cell, proton exchange between the Schiff base and the external medium takes place via an alternative (slower) cytoplasmic channel. As outlined above, it is interesting that the effect of replacing Asp^{85} by a neutral residue differs completely from that of neutralizing Asp^{85} by protonation. Thus, the latter process (in the case of pigment III) does not appear to affect the rate of proton translocation via the channel.

The above considerations are obviously relevant to the exposure direction of the Schiff base, a problem which is of primary importance in the proton pump mechanism (Kalisky et al., 1981). By performing pH jumps in bacteriorhodopsin selectively oriented in vesicle systems, we have shown that the (Asp^{85}) counterion is exposed (to protons) to the outside of the cell (Druckmann et al., 1985). This is consistent with the structural model of bR (Henderson et al., 1990) that places Asp^{85} in an extracellular proton release domain. The question arises as to whether the fact that Asp^{85} is exposed to the outside also implies that the Schiff base has the same exposure direction. Our present observations, as well as those of Kataoka et al. (1994), indicate that this might actually be the case. It will be interesting to verify this conclusion, carrying out pH-jumps experiments in which the Schiff base is directly titrated in oriented membranes of artificial bR, analogously to the experiments performed in the case of the titration of Asp^{85} (Druckmann et al., 1985).

We finally consider the implications of the present conclusions (regarding the titrations of the Schiff base and of Asp^{85} in the unphotolyzed pigment) to the mechanism of the light-induced proton pump in bacteriorhodopsin. It appears to be well established [see reviewing discussion by Lanyi (1993)] that above pH $\simeq$ 6 the proton release to the outside of the cell during the photocycle takes place on a $\sim 10^{-5}$ s time scale. The translocated proton originates from a protein residue (or a H-bonded complex), XH, characterized by an apparent $pK_a \simeq 6$, which is neither the Schiff base nor Asp^{85} (Zimányi et al., 1992). Similarly to the behavior of the Schiff base in the dark, we may tentatively assume that XH behaves like a free acid/base with respect to eq 6. Using the photocycle parameters $k_d \simeq 10^5$ s^{-1} (Scherrer et al., 1994) and $K \simeq 10^{-6}$ (Zimányi et al., 1992), eq 6 yields $k_a = \sim 10^{11}$ M^{-1} s^{-1}. Thus, both protonation and deprotonation of XH during the photocycle are much faster than those of the Schiff base and of Asp^{85} in the dark. Especially, the reprotonation rate constant of XH is essentially diffusion controlled (10^{11} M^{-1} s^{-1}), as compared to $k_a \sim 10^7$ M^{-1} s^{-1} in the case of the Schiff base. These conclusions imply that, at variance with the Schiff base and Asp^{85} (in the dark), during the photocycle XH is highly exposed to the outside medium, behaving in every respect (including the diffusion-limited value of k_a) like a free acid/base in homogeneous aqueous solutions. Below pH $\simeq$ 6 (when XH cannot translocate a proton) proton uptake takes place before release. It appears that the latter process occurs directly from Asp^{85}, on a $\sim 10^{-2}$ s time scale [see Lanyi (1993)]. It is interesting that this rate corresponds to a photocycle deprotonation constant of $k_d \simeq 100$ s^{-1}, which is of the same order of magnitude as that of the deprotonation of Asp^{85} in the dark titrations of native (unphotolyzed) bR, R82Q bR, and aromatic bR (see Table 2). In other words, the deprotonation channels of Asp^{85} in the dark and presumably in the (low pH) photocycle appear to be very similar. As mentioned above, all such channels are considerably less effective than that which links the XH group to the outside. It is therefore likely that XH is in a highly exposed location close to, or on, the membrane surface.

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