Titration of the Bacteriorhodopsin Schiff Base Involves Titration of an Additional Protein Residue†

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ABSTRACT: The retinal protein protonated Schiff base linkage plays a key role in the function of bacteriorhodopsin (bR) as a light-driven proton pump. In the unphotolyzed pigment, the Schiff base (SB) is titrated with a pK_a of ∼13, but following light absorption, it experiences a decrease in the pK_a and undergoes several alterations, including a deprotonation process. We have studied the SB titration using retinal analogues which have intrinsically lower pK_a's which allow for SB titrations over a much lower pH range. We found that above pH 9 the channel for the SB titration is perturbed, and the titration rate is considerably reduced. On the basis of studies with several mutants, it is suggested that the protonation state of residue Glu204 is responsible for the channel perturbation. We suggest that above pH 12 a channel for the SB titration is restored probably due to titration of an additional protein residue. The observations may imply that during the bR photocycle and M photointermediate formation the rate of Schiff base protonation from the bulk is decreased. This rate decrease may be due to the deprotonation process of the "proton-releasing complex" which includes Glu204. In contrast, during the lifetime of the O intermediate, the protonated SB is exposed to the bulk. Possible implications for the switch mechanism, and the directionality of the proton movement, are discussed.

The light-induced proton pump in bacteriorhodopsin (bR),[1] initiated by the absorption of a photon which leads to transmembrane electrochemical potential, is probably the best characterized ion pump. At the core of the pump mechanism in bR are light energy absorption and initial energy storage. These primary events are followed by structural transformations in the retinal chromophore and in the protein which are coupled to the vectorial proton translocation (for a recent review, see ref *1*).

Bacteriorhodopsin is the integral protein of the purple membrane of *Halobacterium salinarum*. It is composed of seven transmembrane helical segments enclosing the binding pocket for the all-*trans* retinal chromophore. The latter is bound to Lys216 via a protonated Schiff base (PSB) linkage. Light absorption initiates a multistep reaction cycle with several distinct spectroscopic intermediates: J_{625}, K_{590}, L_{550}, M_{412}, N_{560}, and O_{640}. It is well established that retinal in K_{590} has a 13-cis configuration (*2, 3*). Deprotonation of the protonated Schiff base takes place during the L to M transition, which is accompanied by protonation of Asp85 and the appearance of a proton at the extracellular surface. The Schiff base is reprotonated during the M to N transition from the proton donor Asp96, which is finally reprotonated from the cytoplasmic side during recovery of the initial state of bR. More details about the molecular alterations that occur during the photocycle were recently obtained by X-ray studies (for a recent review, see ref *1*). It is thus evident that understanding the factors that control the Schiff base (SB) pK_a, the mechanism for its titration, and its possible exposure to the bulk medium is of primary importance for elucidating the function of bR at the molecular level. By using flow and stationary methods, an acid−base equilibrium between bR (in its dark-adapted form) and a species absorbing at 460 nm was detected (*4*). It was shown that this species is characterized by a deprotonated Schiff base moiety, and the apparent pK_a value for the transition is 13.3 ± 0.3. Later, using an artificial bR pigment derived from a chromophore with an intrinsically reduced SB pK_a, it was established that the observed pK_a value is directly associated with the Schiff base of bR (*5*). Recently, it was shown that in the unphotolyzed bR, within a restricted pH range of 7−9, the Schiff base moiety behaves like a free acid or base in solution with respect to the relation between the kinetic rate parameters and the equilibrium constant (*6*).

In our work, we have studied the SB titration mechanism of the wild type (WT) and several mutants of bR using artificial pigments derived from chromophores which have intrinsically reduced pK_a's (Scheme 1). Our data indicate that above pH 9 the channel for the SB titration is significantly disturbed, and the titration rate is considerably decreased. The channel is completely blocked in the E204Q mutant. It is argued that the SB titration channel may as well

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[1] Abbreviations: bR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PSB, protonated Schiff base; SB, Schiff base; WT, wild type.

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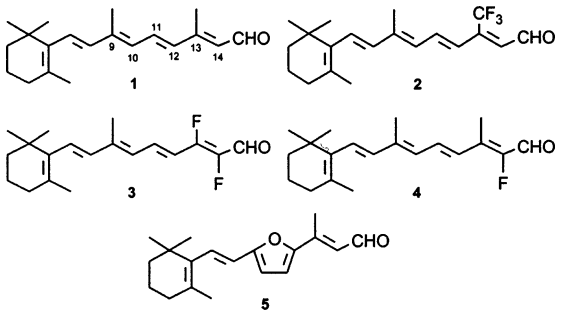
Scheme 1: All-*trans*-Retinal (**1**) and Synthetic Retinal Analogues



be perturbed during the photocycle. Following PSB deprotonation and a proton release, the channel for the Schiff base (SB) reprotonation from the bulk is significantly perturbed, preventing SB reprotonation from the extracellular side.

MATERIALS AND METHODS

Sample Preparation. BR and its mutants were purified using published methods (*7*). The apoprotein was prepared as described elsewhere (*8*), as well as the synthesis of the retinal analogues (*5*, *9*). Artificial pigments were prepared by incubating the appropriate apoprotein with 1.5−2 equiv of chromophore and 30 mM phosphate buffer at pH 7 (room temperature).

Titration Experiments. Titrations were performed in the dark by addition of small amounts of base (typically, 2 μL of 0.05−5 M NaOH, as appropriate) to the dark-adapted pigment, followed by absorption measurements. The absorption at the λ_{max} of the PSB was plotted against the pH, and the data were fitted to a modified equation of the form

$$A = \frac{a}{1 + 10^{n(\mathrm{pH}-\mathrm{p}K_{a1})}} + \frac{1-a}{1 + 10^{n(\mathrm{pH}-\mathrm{p}K_{a2})}}$$

where A is the normalized absorbance, a is the fraction of untitrated PSB, and n is the cooperativity index (Hill index), in the cases of two pK_a values. The second term was omitted if only one titration was observed.

*Determination of the pK_a of Chromophore **3** SB.* The apparent pK_a of chromophore **3** (Scheme 1) Schiff base (SB) was determined in an ethanol/water (1:1) solution. The chromophore **3** *n*-butylamine Schiff base was dissolved in an ethanol/water (1:1) solution and mixed with an appropriate buffer. Formation of the red-shifted protonated Schiff base was monitored by absorption spectroscopy.

Photocycle Intermediates. The pigments' photochemically induced intermediates were monitored by irradiating the sample at 25 °C with a flash lamp using an appropriate cutoff filter (Schott), and following the formation and decay of the photointermediates with an Agilent 4583 diode array spectrophotometer.

FT Raman Measurements. Samples were prepared by washing the pigment with deionized distilled water or with D_2O in the dark. Measurements were carried out with a Bruker RFS100/S Fourier transform Raman spectrometer (Bruker), equipped with a 1064 nm Nd:YAG laser system. The excitation laser was set up to 900 mW. Raman spectra were recorded in a backscattering arrangement, averaging 100 scans at 2 cm^{-1} resolution.

RESULTS

SB Titrations. Titrations of wild-type (WT) and mutants bR's (E204Q, E194C, D96N, and R82Q) and of artificial pigments derived from synthetic chromophores reconstituted with various bacterioopsins were carried out. The Schiff bases of the synthetic retinals (Scheme 1) have intrinsically reduced pK_a's. The titration of the SB was followed by monitoring the pigment absorption which shifts to ∼400 nm in the artificial pigments in contrast to that of WT bR which shifts at high pH mostly to a species absorbing at 460 nm. The titration process was confirmed (as opposed to decomposition) by an immediate pigment recovery following rapid pH decrease, indicating process reversibility. It was previously shown that the decrease in the SB pK_a of synthetic retinal analogues, bearing electron-withdrawing groups, is reflected in the SB pK_a of the respective pigments (*5*, *10*). Therefore, these artificial pigments enable titration at a relatively low pH range, preventing titration of additional protein residues at high pH. Titration of 13-CF_3-bR (derived from chromophore **2**) indicated that only ca. 70% of the pigment was titrated with a pK_a of ∼8.8, whereas the remaining fraction had a pK_a of >12 (Figure 1A and Table 1). Similar behavior was detected in the presence of a high salt concentration (1 M NaCl or 1 M Na_2SO_4). The most plausible explanation for the two titration phases involves an additional protein residue which participates in the process. In an attempt to pinpoint this residue, we examined 13-CF_3 artificial pigments derived from mutants R82Q, D96N, E204Q, and E194C. Both 13-CF_3-D96N and 13-CF_3-R82Q exhibited two titration transitions like WT; however, with a somewhat lower pK_a for both transitions (Figure 1B and Table 1). An especially lowered pK_a value was observed for 13-CF_3-D96N. We attribute this observation to alteration of the hydrogen bonding network in the SB vicinity caused by the Asp96 mutation. A completely different behavior was observed for pigment 13-CF_3-E204Q in which the PSB could not be deprotonated below pH 12 (Figure 2A). Since it was suggested that the "proton-releasing complex" in bR is comprised of both Glu204 and Glu194 residues, we have examined the 13-CF_3-E194C pigment. In contrast to 13-CF_3-E204Q, 13-CF_3-E194C exhibited a trend similar to that of 13-CF_3-bR. Namely, the titration curve indicated two transitions with pK_a values of 8.4 and 10.9 (Table 1 and Figure 2A).

13,14-Difluororetinal **3** and 14-fluororetinal **4** represent additional examples of chromophores which have SB pK_a values intrinsically reduced relative to that of retinal SB in solution by ca. 4 and 2.5 pK_a units, respectively (*11*). Titration of the artificial pigments derived from chromophores **3** and **4** indicated that only ∼60% of 13,14-diF-bR was titrated with a pK_a of 8.5 and ∼40% of 14-F-bR with pK_a of 9.3, while the remaining fractions had pK_a's above 12 (Figure 2B and Table 1). Similar results were obtained in the presence of high salt concentrations (Table 1) which offset the effect of the membrane surface potential on the pK_a (*12*−*14*), and indicate that all protein residues involved in the titration are similarly affected by salt. It was previously shown that the effect of the membrane surface potential is diminished by a partial delipidation process via treatment with CHAPS (*15*, *16*). Therefore, in a partially delipidated form, the pK_a of Asp85 is reduced to a value

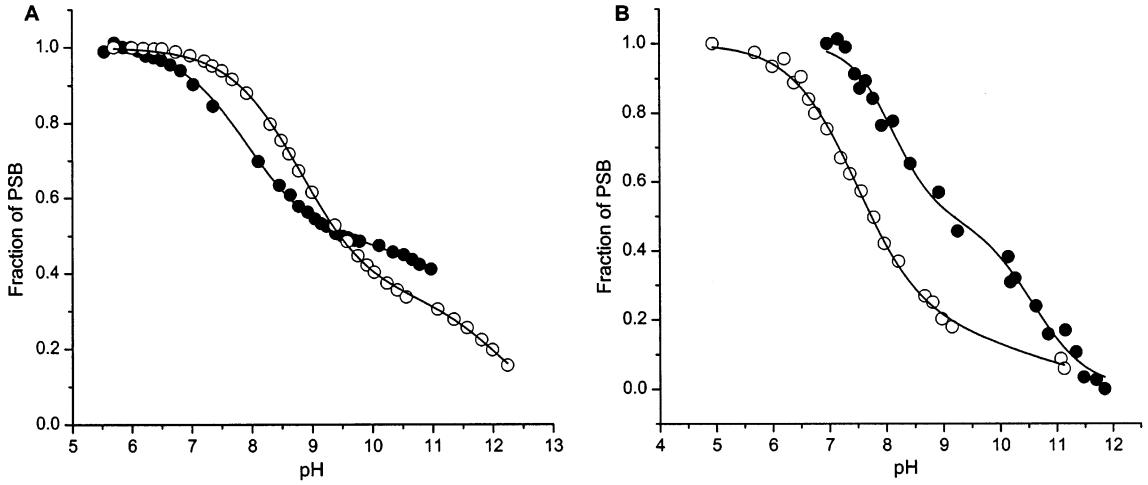


FIGURE 1: (A) Titration of 13-CF_3-bR derived from chromophore **2** in 30 mM phosphate buffer (○) and in 1 M NaCl (●). (B) Titration of 13-CF_3-D96N (○) and 13-CF_3-R82Q (●). Titrations were carried out in the presence of 30 mM phosphate buffer and 20 mM NaCl. In all cases, solid lines represent the best fit to the modified Henderson−Hasselbalch equation.

Table 1: Calculated pK_a Values of SB Titrations of Artificial Pigments (WT and mutants) Derived from Synthetic Retinal Analogues

	chromophore **2**			chromophore **3**			chromophore **4**			chromophore **5**		
	pK_{a1}[a]	a[b]	pK_{a2}[a]	pK_{a1}[a]	a[b]	pK_{a2}[a]	pK_{a1}[a]	a[b]	pK_{a2}[a]	pK_{a1}[a]	a[b]	pK_{a2}[a]
WT	8.8	0.66	12.2	8.5	0.63	11.6	9.3	0.43	12.4	9.4	0.56	10.3
WT (1 M NaCl)	7.9	0.50	12.0	8.0	0.42	12.0	8.2	0.25	12.3	8.7	0.74	11.5
WT (1 M Na_2SO_4)	7.9	0.55	12.0				7.8	0.32	12.1	8.9	0.69	12.0
WT (CHAPS)		–		8.1	0.26	>12						
D96N	7.5	0.79	10.3	8.2	–	–	8.1	–	–	8.3	0.60	9.4
R82Q	8.1	0.50	10.6	7.9	–	–	8.9	0.49	11.5	9.1	–	–
E204Q	>12	–	–	>12	–	–	>12	–	–	7.9	–	–
E194C	8.4	0.74	10.9	7.8	0.35	8.8				8.0	0.40	9.0

[a] The error for pK_a values is ±0.1. [b] a is the fraction of pigment titrated with the lower pK_a.

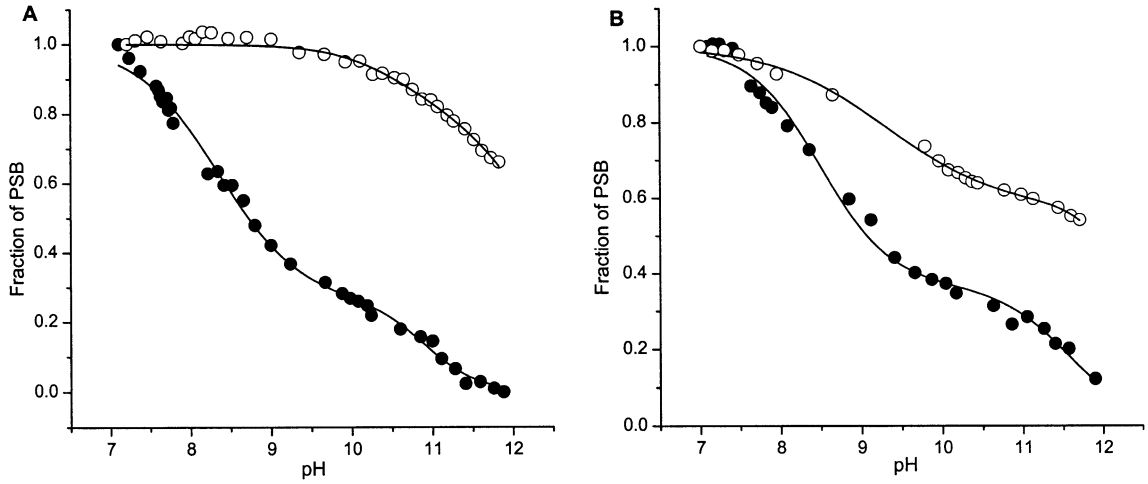


FIGURE 2: (A) Titration of pigments 13-CF_3-E204Q (○) and 13-CF_3-E194C (●). Conditions were similar to those described in the legend of Figure 1B. (B) Titration of pigments 13,14-diF-bR (○) and 14-F-bR (●). Titrations were performed in 30 mM phosphate buffer. In all cases, solid lines represent the best fit to the modified Henderson−Hasselbalch equation.

resembling its pK_a at high salt concentrations in intact membranes. We have checked this effect on the SB titration of 13,14-diF-bR and observed a behavior similar to that of at high salt concentrations (Table 1). Namely, the partial delipidation process eliminated the membrane surface potential effect on the SB titration.

Pigments of E204Q reconstituted with chromophores **3** and **4** (13,14-diF-E204Q and 14-F-E204Q) could not be titrated below pH 12 despite the fact that the intrinsic SB pK_a was significantly reduced (Table 1). These results support those obtained for pigment 13-CF_3-E204Q derived from 13-CF_3 retinal **2**. In contrast, 13,14-diF-E194C exhibited complete SB titration at low pH (pK_{a1} = 7.8, and pK_{a2} = 8.8) without the remaining untitrated fraction (Figure 3A). It is plausible that the pK_a of the protein residue involved in the titration of 13,14-diF-bR is increased in the E194C mutant, and does not influence the titration of 13,14-diF-E194C. Pigments 13,14-diF-D96N and 14-F-D96N (Figure 3A and Table 1) did not exhibit a clear separation for two titration transitions. However, the titration was spread over a wide pH range (~3

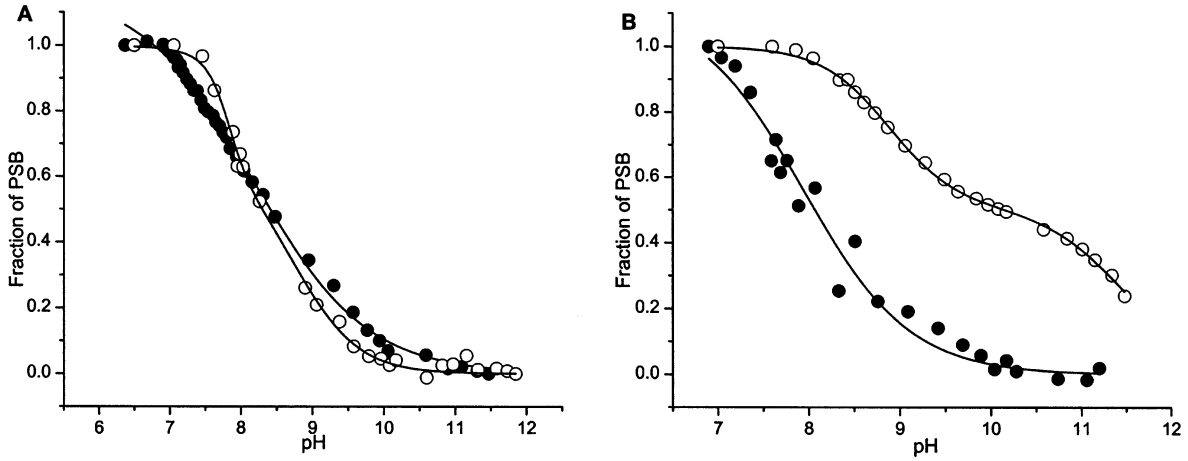


FIGURE 3: (A) Titration of pigments 13,14-diF-E194C (○) and 13,14-diF-D96N (●). (B) Titration of pigments 13,14-diF-R82Q (●) and 14-F-R82Q (○). Conditions were as described for Figure 1B. In all cases, solid lines represent the best fit to the modified Henderson−Hasselbalch equation.

units). The titration of 14-F-D96N revealed an unusually low pK_a (8.1), which suggests a perturbation in the protonated SB environment. Therefore, although located in the cytoplasmic side, the D96N mutation may affect the hydrogen bonding network in the SB vicinity and perturbs its pK_a. The exact mechanism should be studied in future work. Mutant R82Q exhibited two transitions in both 13-CF_3-R82Q (Table 1) and 14-F-R82Q (Figure 3B) but not in 13,14-diF-R82Q.

The pK_a of the SB is significantly decreased due to structural alterations (*17*). Substitution of Asp85, which serves as a major component in protonated SB stabilization, with a neutral residue reduces the SB pK_a to ~8 (*18*, *19*). It was noted previously (*19*) that the titration of the D85N mutant involves two components. In keeping with this observation, we have observed that the titration of the D85N mutant has a fraction of ~15% which is titrated with a pK_a of >12 (data not shown). This observation further supports our finding that an additional protein residue is involved in the SB titration once the SB pK_a is ca. 8. Another possibility of altering the SB pK_a is via conformational rearrangements in the retinal binding site. Such an effect can be introduced by employing the artificial pigment 11,14-epoxy-bR (derived from retinal analogue **5)** which enforces structural alterations in the retinal binding site due to its unusual structure (*17*). These alterations increase the pK_a of Asp85 and decrease that of the SB. Spectroscopic titrations of artificial pigments derived from chromophore **5** and a series of mutants indicate two titration transitions whose pK_a values are ca. 9 and 10.5−12 (Table 1). We note the following observations for the artificial pigments derived from chromophore **5**. (1) Like the results (described above) obtained for mutant D96N, this mutation decreases the SB pK_a in the artificial pigment 11,-14-epoxy-D96N (Table 1). (2) The titration of 11,14-epoxy-R82Q could not be separated into two transitions. (3) Interestingly, unlike the (described above) fluorinated artificial pigments derived from mutant E204Q, 11,14-epoxy-E204Q did exhibit a low SB pK_a (7.9) which obeys the normal Henderson−Hasselbalch titration.

Slow Titrations and H−D Exchange. The above-described titrations were carried out by measuring the absorption immediately following pH adjustment. The two unusual transitions revealed in most of the titrations may originate from an interaction with another protein residue which changes its protonation state, thereby affecting the SB pK_a. However, another possibility involves blocking of the SB titration channel altogether due to deprotonation of a protein residue, which possibly could be identified as Glu204. In the latter case, the SB titration rate would be reduced along with the protein residue titration rate. However, complete blocking will be prevented due to a constant presence of a protonated fraction of the protein residue. To distinguish between the two possibilities, we have followed the absorption of pigments 13-CF_3-bR and 13,14-diF-bR at pH 9 in which the titration was inhibited. We have observed that the titration proceeded at a very slow rate (few hours) until the expected titrated fraction for the normal Henderson−Hasselbalch equation was reached. We note that during this slow process a slow Schiff base hydrolysis reaction occurred. This reaction prevented a completely reversible titration, and only ~85% of the titrated fraction was reversible after ca. 4 h. However, these results clearly indicate that the titration of the protein residue blocks a channel for the SB titration. To support these results and identify the protein residue which perturbs the titration channel, we have followed the absorption of 13-CF_3-E204Q and 13,14-diF-E204Q at different pH values below 10. We did not observe any titration even after 12 h. This result supports the view that deprotonation of Glu204 blocks the SB titration channel since, obviously, titration of Glu204 was eliminated in E204Q.

In contrast to the SB titration, H−D exchange at the protonated SB of E204Q could be achieved at pH 7. This was demonstrated by monitoring the C═N stretch of the E204Q mutant using the FT Raman technique. A characteristic shift from 1640 to 1620 cm^{-1} was observed following exchange of H_2O with D_2O (data not shown).

SB Titrations in a Photocycle Intermediate. Previous studies have indicated that the rate of decay of the E204Q O photocycle intermediate to the initial pigment is decreased relative to that of the native pigment. This effect is probably due to a lack of an intramolecular proton acceptor (Glu204−Glu194−water complex) for the Asp85 proton (*20*). Spectroscopic titration of 14-F-bR suggested that the pK_a of the SB in the O-like intermediate is ca. 11, and was reduced to ca. 9 in 14-F-E204Q (*21*). Our above-described studies suggested that the protonation state of Glu204 affects the proton channel for the SB titration in the ground dark state.

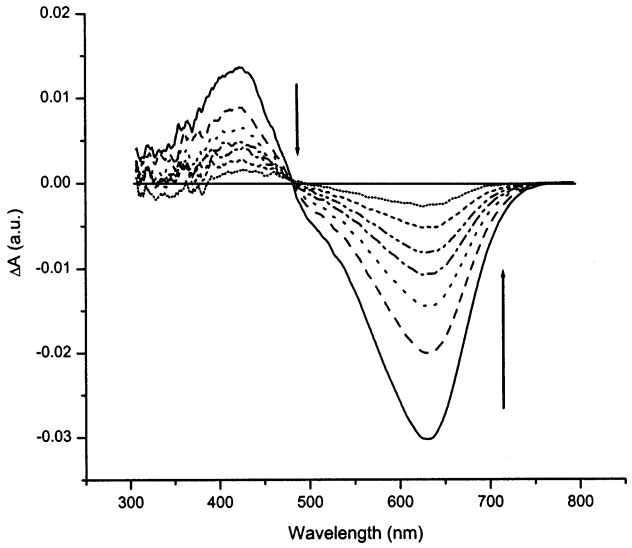


FIGURE 4: Photocycle of pigment 13-CF_3-E204Q. The pigment was irradiated at pH 7 with a cutoff filter of 540 nm, followed by monitoring the decay of the long-lived intermediate. Arrows denote progression of spectra 0, 7, 12, 17, 22, 34, and 77 s after irradiation. Difference spectra were obtained by subtracting the spectrum before irradiation from the spectra recorded after irradiation. Similar results were obtained for pH values of 5 and 9.

Mutation of Glu204 closes this channel at neutral pH and prevents SB titration. To check the validity of this effect during the lifetime of the photochemically induced O intermediate, and whether the SB of the O intermediate can be titrated at pH <7, we have studied the photocycle of 13-CF_3-E204Q. It is expected that the O intermediate of this pigment will have a very low SB pK_a due to both fluorine substitution and Glu204 mutation. Irradiation of pigment 13-CF_3-E204Q with a flash lamp at pH 7 gave a long-lived M-like intermediate which thermally decayed to the pigment with a k of 0.05 s^{-1} (Figure 4). The decay rate of the M intermediate in the photocycle of bR is significantly increased following blue light irradiation. In contrast, the decay rate of the M-like intermediate derived from 13-CF_3-E204Q was not affected by blue light irradiation. Illumination of 13-CF_3-E204Q at pH 5 similarly produced an M-like intermediate which thermally decayed to the pigment with a k of 0.29 s^{-1}. The results may imply that the SB of the 13-CF_3-E204Q O intermediate has a pK_a of <5.

DISCUSSION

Substitution of the retinal polyene with electron-withdrawing groups such as fluorine enables titration of the Schiff base at relatively low pH, preventing high pH values which may cause titration of additional protein residues. This study clearly indicates a complex titration of the Schiff base linkage which is associated with more than one protein residue. It was previously suggested (*22*) that the titration of Asp85 which controls the purple → blue transition of bR is complex since this residue interacts with the proton-releasing complex located in the extracellular part of the protein. The protonation state of this complex affects and modifies the pK_a of Asp85, leading to a titration which involves several transitions. However, this model does not apply to the SB titration since we have found that at pH ca. 9 the titration of the Schiff base is not completely inhibited but its rate is significantly decreased. Therefore, the SB pK_a is not altered, but rather, its titration is perturbed. We suggest that deprotonation of a protein residue whose pK_a is ca. 9 significantly affects the channel for the SB titration. At pH >11, other protein residues are deprotonated and reopen the titration channel or, alternatively, allow for titration via new channels. Therefore, it appears that in WT pigment the pK_a of the Schiff base is indeed ca. 13, as demonstrated previously (*4*, *5*). The channels for the WT SB titration (which were blocked by titration of a protein residue with a pK_a of ca. 9) are reopened by titration of other protein residues at high pH. Interestingly, at high pH (ca. 9.5), Asp85 is titrated (*23*), whereas the titration of the SB is inhibited. This may indicate that at high pH the two residues are not titrated via an identical channel.

Identification of the Protein Residue Responsible for Blocking SB Titration. The titration of the SB is completely inhibited in mutant E204Q at pH <12 which indicates that the mutation blocked the channel for the SB titration. The titration is possible only at high pH due to deprotonation of additional groups which reopen a new channel for titration. We suggest that residue Glu204 is an important component in the SB titration channel. It is tempting to identify Glu204 as the residue with a pK_a of ca. 9 which inhibits the SB titration upon its deprotonation. A similar pK_a value was previously suggested for Glu204 (*23*, *24*). While in mutant E204Q titration of the SB linkage is blocked, H−D exchange still takes place. It was previously shown (*25*) that the time scale of H−D exchange of the SB in bR is considerably faster than that predicted by mechanisms based on base-catalyzed deprotonation followed by reprotonation. Such mechanisms were therefore excluded, leading to an alternative mechanism based on direct interaction of a water molecule with the Schiff base in a concerted exchange step. The inapplicability of the base-catalyzed mechanism was later supported by the rate measured for Schiff base deprotonation which was shown (*6*) to be slower by a few orders of magnitude than H−D exchange. The concerted mechanism, involving a water molecule interacting with the Schiff base, is based on H−D exchange between the external aqueous medium and the exchanging water molecule, followed by exchange between the latter and the Schiff base. The results presented here, indicating that E204Q mutation blocks the channel for SB titration but not for H−D exchange, support different mechanisms for the two processes.

Deprotonation of Glu204 obviously introduces a negative charge; however, its substitution with asparagine does not change the charge balance, but still completely blocks the SB titration. Therefore, it is possible that deprotonation of Glu204 or its substitution with asparagine modifies the hydrogen bonding network which is crucial for an effective SB titration channel. Indeed, X-ray studies of mutant E204Q indicated considerable rearrangement of water molecules in the vicinity of Gln204 (*26*). Alternatively, it was proposed that Glu204 is deprotonated in the ground state (*27*), and its proton is stabilized by a water cluster. Increasing the pH or Glu204 substitution can both lead to deprotonation of the water cluster and significantly modify the SB titration channel. We note that while the E204Q mutation completely blocked the SB titration, deprotonation of Glu204 considerably reduces the titration rate but does not block it completely. We attribute the slow titration to a minor protonated fraction persisting in the titration mixture.

It was suggested that the proton-releasing complex in bR consists of residues Glu204 and Glu194 and water, since mutation of either Glu204 or Glu194 significantly affected the proton release process (*28*, *29*). However, while substitution of E204 with asparagine completely blocked the SB titration, substitution of Glu194 (with cysteine) did not affect it. This result further indicates the important role that residue Glu204 plays in affecting the channel for SB titration. The sensitivity of the channel effectiveness to the protein conformation and the structure of the hydrogen bonding network is demonstrated as well by studies of pigments derived from chromophore **5**. This chromophore is significantly distorted due to its furan ring which induces protein conformation alteration in the SB environment, and increases the pK_a of Asp85 (*17*). However, mutation of Glu204 with asparagine did not block the SB titration in 11,14-epoxy-E204Q, probably since the distorted chromophore has modified the titration channel. The titration of 11,14-epoxy-E204Q SB further supports the suggestion that titration of Glu204 significantly decreases the rate of SB titration since in this pigment the titration obeys Henderson−Hasselbalch titration involving only the SB moiety (Table 1).

Implications for bR Function. Following light absorption, the protonated SB, which is exposed to the extracellular side (*6*, *30*), transfers its proton to Asp85. This event is followed by a proton release from the proton-releasing complex associated with Glu204, Glu194, and water molecules located on the extracellular side. To complete the pumping process, the Schiff base has to undergo a "switch" process which exposes it to the cytoplasmic side. Several mechanisms have been suggested for the switch process: (1) reorientation of the Schiff base nitrogen from the extracellular side to the cytoplasmic side due to a change in retinal curvature following deprotonation (*31*, *32*), or following alterations in electrostatic interactions, including the movement of water molecules (*33*−*35*); (2) deformation of helix C during the photocycle (*36*); (3) movement of a hydroxyl anion from the extracellular to the cytoplasmic side (*37*); and (4) an increase in the pK_a of Asp85 during the photocycle which enforces protonation from Asp96 (*23*).

Although the exact switch mechanism has not been elucidated, it is clear that SB reprotonation from the extracellular side should be prevented during this process. One mechanism is associated with a very low SB pK_a which will prevent reprotonation. The pK_a of the SB in the dark state is ca. 13 (*4*, *5*), but its M intermediate value is unknown. We note that the transfer of the proton from the protonated SB to Asp85 does not necessarily imply that the SB pK_a is considerably reduced. The proton transfer would be controlled by the energy difference between the protonated SB−Asp85 ion pair and the Schiff base−Asp85 (protonated) pair rather than by the equilibration of the protonated SB with the outside medium. Therefore, an additional mechanism for preventing SB reprotonation from the extracellular side is via blocking the channel for SB reprotonation, or significantly reducing the rate of the process. It is tempting to suggest that deprotonation of the proton-releasing complex significantly reduces the rate of the SB titration, and prevents its reprotonation irrespective of its pK_a. It was suggested (*6*) that during the photocycle the proton-releasing complex is highly exposed to the outside medium, behaving like a free acid or base in a homogeneous aqueous solution. Therefore, deprotonation of the proton-releasing complex is much faster than titration of the SB. The deprotonation process considerably reduces the SB titration rate and practically blocks its exposure to the extracellular side. In this respect, we note that the pK_a of the SB was determined in the late M state of mutant D96N to be 8.2−8.3 (*38*). This value might be true also for the wild type and raises the question of why the SB is not protonated from the extracellular side at this stage but captures a proton from an internal residue (Asp96). Blocking the channel for SB titration via deprotonation of the proton-releasing complex may provide an explanation for this phenomenon.

As suggested previously, the SB pK_a of the O intermediate is ca. 11, since deprotonation of the protonated SB was detected in artificial pigments which have a long-lived O intermediate (*21*). This pK_a value was further confirmed in this work in the 13-CF_3-E204Q pigment. The pK_a of the retinal chromophore SB was intrinsically reduced by ca. 5 pK_a units, which is reflected in the SB pK_a of the O intermediate. The pK_a is further reduced due to the mutation, and irradiation of the pigment yielded a stable O intermediate which consists of a retinal SB (rather than a protonated SB) even at pH 6. This result implies that the O intermediate SB is exposed to the bulk solution and can be deprotonated even though residue Glu204 is deprotonated or mutated. The decay of the O intermediate to the bR initial state is associated with a proton transfer from Asp85 to the proton-releasing complex possibly through Asp212 (*39*, *40*). In mutant E204Q, the proton of Asp85 is directly transferred to the bulk (*20*). Therefore, Asp85 is probably exposed to the extracellular side in the WT as well, and may connect the protonated SB to the bulk medium. It is plausible that during the photocycle the SB experiences different levels of exposure to the bulk medium.

The thermal decay of the O intermediate in 13-CF_3-E204Q is unusually slow probably since its chromophore consists of a SB rather than a protonated SB. It is conceivable that a major driving force for the deprotonation of Asp85 and transfer of its proton to the proton-releasing complex or to the bulk medium is associated with the presence of a positively charged Schiff base that participates in the decrease in the pK_a of Asp85. Since this mechanism is not operative in 13-CF_3-E204Q, a (slow) protein conformation alteration is required to reduce the pK_a of Asp85 and induce its deprotonation, thereby increasing the SB pK_a and inducing its protonation.

REFERENCES

1. Lanyi, J. (2004) Bacteriorhodopsin, *Annu. Rev. Physiol. 66*, 665−688.
2. Honig, B., Ebrey, T., Callender, R., Dinur, U., and Ottolenghi, M. (1979) Photoisomerization, energy storage, and charge separation: A model for light energy transduction in visual pigments and bacteriorhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503−2507.
3. Braiman, M., and Mathies, R. (1982) Resonance Raman spectroscopy of bacteriorhodopsin's primary photoproduct: Evidence for a distorted 13-cis retinal chromophore, *Proc. Natl. Acad. Sci. U.S.A. 79*, 403−407.
4. Druckmann, S., Ottolenghi, M., Pande, A., Pande, J., and Callender, R. (1982) Acid−base equilibrium of the Schiff base in bacteriorhodopsin, *Biochemistry 21*, 4953−4959.
5. Sheves, M., Albeck, A., Friedman, N., and Ottolenghi, M. (1986) Controlling the pK_a of the bacteriorhodopsin Schiff base by use of artificial retinal analogues, *Proc. Natl. Acad. Sci. U.S.A. 83*, 3262−3266.

6. Druckmann, S., Ottolenghi, M., Rousso, I., Friedman, N., and Sheves, M. (1995) Time-resolved titration of the Schiff base and of the Asp-85 residue in artificial bR, *Biochemistry 34*, 12066−12074.
7. Oesterhelt, D., and Stoeckenius, W. (1974) Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane, *Methods Enzymol. 31*, 667−678.
8. Aharoni, A., Weiner, L., Lewis, A., Ottolenghi, M., and Sheves, M. (2001) Nonisomerizable non-retinal chromophores initiate light-induced conformational alterations in bacterioopsin, *J. Am. Chem. Soc. 123*, 6612−6616.
9. Asato, A. E., and Liu, R. S. H. (1986) The preparation of vicinal difluoroolefinic carbonyl compounds and their application to the synthesis of difluororetinal analogs, *Tetrahedron Lett. 27*, 3337−3340.
10. Govindjee, R., Balashov, S., Ebrey, T., Oesterhelt, D., Steinberg, G., and Sheves, M. (1994) Lowering the intrinsic pK_a of the chromophore's Schiff base can restore its light induced deprotonation in the inactive Tyr-57 to Asn mutant of bR, *J. Biol. Chem. 269*, 14353−14354.
11. Steinberg, G., Sheves, M., Bressler, S., and Ottolenghi, M. (1994) Factors affecting the formation of an M-like intermediate in the photocycle of 13-cis-bacteriorhodopsin, *Biochemistry 33*, 12439−12450.
12. Szundi, I., and Stoeckenius, W. (1989) Surface pH controls purple-to-blue transition of bacteriorhodopsin, *Biophys. J. 56*, 369−383.
13. Chang, C. H., Chen, J. G., Govindjee, R., and Ebrey, T. (1985) Cation binding by bacteriorhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 82*, 396−400.
14. Eliash, T., Weiner, L., Ottolenghi, M., and Sheves, M. (2001) Specific binding sites for cations in bacteriorhodopsin, *Biophys. J. 81*, 1155−1162.
15. Szundi, I., and Stoeckenius, W. (1987) Effect of lipid surface charges on the purple-to-blue transition of bacteriorhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 84*, 3681−3684.
16. Eliash, T., Ottolenghi, M., and Sheves, M. (1999) The titrations of Asp-85 and of the cation binding residues in bacteriorhodopsin, *FEBS Lett. 447*, 307−310.
17. Rousso, I., Friedman, N., Sheves, M., and Ottolenghi, M. (1995) pK_a of the protonated Schiff base and aspartic 85 in the bacteriorhodopsin binding site is controlled by a specific geometry between the two residues, *Biochemistry 34*, 12059−12065.
18. Turner, G., Miercke, L., Thorgeirsson, T., Kliger, D., Betlach, M., and Stroud, R. (1993) Bacteriorhodopsin D85N: Three spectroscopic species in equilibrium, *Biochemistry 32*, 1332−1337.
19. Brown, L., Bonet, L., Needleman, R., and Lanyi, J. (1993) Estimated acid dissociation constants of the Schiff base, Asp-85, and Arg-82 during the bacteriorhodopsin photocycle, *Biophys. J. 65*, 124−130.
20. Misra, S., Govindjee, R., Ebrey, T., Chen, J. M., and Crouch, R. (1997) Proton uptake and release are rate-limiting steps in the photocycle of the bacteriorhodopsin mutant E204Q, *Biochemistry 36*, 4875−4883.
21. Gat, Y., Friedman, N., Sheves, M., and Ottolenghi, M. (1997) Interaction between Asp-85 and the proton-releasing group in bacteriorhodopsin. A Study of an O-like photocycle intermediate, *Biochemistry 36*, 4135−4148.
22. Balashov, S., Govindjee, R., Kono, M., Imasheva, E., Lukashev, E., Ebrey, T., Crouch, R., Menick, D., and Feng, Y. (1993) Effect of the arginine 82 to alanine mutation in bacteriorhodopsin on dark adaptation, proton release and photochemical cycle, *Biochemistry 32*, 10331−10343.
23. Balashov, S., Imasheva, E., Govindjee, R., and Ebrey, T. (1996) Titration of Aspartate 85 in Bacteriorhodopsin: What it says about chromophore isomerization and proton release, *Biophys. J. 70*, 473−481.
24. Dioumaev, A. K., Richter, H. T., Brown, L., Tanio, M., Tuzi, S., Saito, H., Kimura, Y., Needleman, R., and Lanyi, J. (1998) Existence of a proton transfer chain in bacteriorhodopsin: Participation of Glu-194 in the release of protons to the extracellular surface, *Biochemistry 37*, 2496−2506.
25. Doukas, A., Pande, A., Suzuki, T., Callender, R., Honig, B., and Ottolenghi, M. (1981) On the mechanism of hydrogen−deuterium exchange in bacteriorhodopsin, *Biophys. J. 33*, 275−280.
26. Luecke, H., Schobert, B., Cartailler, J. P., Richter, H. T., Rosengarth, A., Needleman, R., and Lanyi, J. (2000) Coupling photoisomerization of retinal to directional transport in bacteriorhodopsin, *J. Mol. Biol. 300*, 1237−1255.
27. Rammelsberg, R., Huhn, G., Lubben, M., and Gerwert, K. (1998) Bacteriorhodopsin's intramolecular proton release pathway consist of a hydrogen-bonded network, *Biochemistry 37*, 5001−5009.
28. Brown, L. S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R., and Lanyi, J. K. (1995) Glutamic acid 204 is the terminal proton release group at the extracellular surface of bacteriorhodopsin, *J. Biol. Chem. 270*, 27122−27126.
29. Balashov, S., Imasheva, E., Ebrey, T., Chen, N., Menick, D., and Crouch, R. (1997) Glutamate 194 to cysteine mutation inhibits fast light induced proton release in bacteriorhodopsin, *Biochemistry 36*, 8671−8676.
30. Kataoka, M., Kamikubo, H., Tokunaga, F., Brown, L., Yamazaki, Y., Maeda, A., Sheves, M., Needleman, R., and Lanyi, J. K. (1994) Energy coupling in an ion pump, *J. Mol. Biol. 243*, 621−638.
31. Subramaniam, S., and Henderson, R. (2000) Molecular mechanism of vectorial proton translocation by bacteriorhodopsin, *Nature 406*, 653−657.
32. Xu, D., Sheves, M., and Schulten, K. (1995) Molecular dynamics study of the M_{412} intermediate of bacteriorhodopsin, *Biophys. J. 69*, 2745−2760.
33. Herzfeld, J., and Tounge, B. (2000) NMR probes of vectoriality in the proton motive photocycle of bacteriorhodopsin: Evidence for an 'electrostatic steering' mechanism, *Biochim. Biophys. Acta 1460*, 95−100.
34. Lanyi, J., and Schobert, B. (2002) Crystallographic structure of the retinal and the protein after deprotonation of the Schiff base: The switch mechanism in the bacteriorhodopsin photocycle, *J. Mol. Biol. 321*, 727−737.
35. Takeda, K., Matsui, Y., Kamiya, N., Adachi, S., Okumura, H., and Kouyama, T. (2004) Crystal structure of the M intermediate of bacteriorhodopsin: Allosteric structural changes mediated by sliding movement of a transmembrane helix, *J. Mol. Biol. 341*, 1023−1037.
36. Royant, A., Edman, K., Ursby, T., Pebay-Peyroula, E., Landau, E. M., and Neutze, R. (2000) Helix deformation is coupled to vectorial transport in the photocycle of bacteriorhodopsin, *Nature 406*, 645−648.
37. Luecke, H. (2000) Atomic resolution structures of bacteriorhodopsin photocycle intermediates: The role of discrete water molecules in the function of this light driven ion pump, *Biochim. Biophys. Acta 1460*, 133−156.
38. Brown, L., and Lanyi, J. (1996) Determination of the transiently lowered pK_a of the retinal Schiff base during the photocycle of bacteriorhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 93*, 1731−1734.
39. Dioumaev, A. K., Brown, L. S., Needleman, R., and Lanyi, J. K. (1999) Fourier Transform Infrared spectra of a late intermediate of the bacteriorhodopsin photocycle suggest a transient protonation of Asp-212, *Biochemistry 38*, 10070−10078.
40. Zscherp, C., and Herberle, J. (1997) Infrared difference spectra of the intermediates L, M, N, and O of the bacteriorhodopsin photoreaction obtained by time-resolved attenuated total reflection spectroscopy, *J. Phys. Chem. B 101*, 10542−10547.

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