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Review

Protonation reactions and their coupling in bacteriorhodopsin

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Abstract

Light-induced changes of the proton affinities of amino acid side groups are the driving force for proton translocation in bacteriorhodopsin. Recent progress in obtaining structures of bacteriorhodopsin and its intermediates with an increasingly higher resolution, together with functional studies utilizing mutant pigments and spectroscopic methods, have provided important information on the molecular architecture of the proton transfer pathways and the key groups involved in proton transport. In the present paper I consider mechanisms of light-induced proton release and uptake and intramolecular proton transport and mechanisms of modulation of proton affinities of key groups in the framework of these data. Special attention is given to some important aspects that have surfaced recently. These are the coupling of protonation states of groups involved in proton transport, the complex titration of the counterion to the Schiff base and its origin, the role of the transient protonation of buried groups in catalysis of the chromophore's thermal isomerization, and the relationship between proton affinities of the groups and the pH dependencies of the rate constants of the photocycle and proton transfer reactions.

Keywords: Proton affinity; Coupling of protonation states; Proton release/uptake; Proton release complex

1. Introduction

Light-induced proton translocation in bacteriorhodopsin (bR) is a multistep process driven by changes of the proton affinities of amino acid side groups. These changes have received much attention and have been treated in several comprehensive reviews [1–5]. In the present review I consider some important aspects of this broad problem developed recently. After the introduction describing the steps and key groups involved in proton transport features of the initial (unphotolyzed) bR that are directly related to its function are described. These include the pK_a of the key groups involved in proton transport and coupling of their protonation states [6–14], the role of the transient protonation of the counterion in catalysis of the chromophore's thermal isomerization [6,8,15], and the role of coupling of the primary proton acceptor with other groups in the photocycle reactions and proton transport [7,8,10]. The second part of the review deals with mechanisms of light-induced proton release and uptake and the relationship between proton affinities of the key groups and the pH dependence of the kinetics of the photocycle reactions and proton transfer [13,16–19]. Mechanisms of light-induced transmembrane proton transport are discussed based on recent structures of bR and its intermediates that provided important insights in the molecular basis of this process [20–31].

Abbreviations: bR, bacteriorhodopsin; PRC, proton release complex, designated also X; BM, blue membrane

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2. Key residues involved in proton transport and the main steps of proton translocation in the photocycle

Light energy transduction in bR proceeds via a cyclic reaction (photocycle) involving a chain of intermediate states which were identified spectroscopically (the subscript shows a position of absorption maximum):

$$bR_{568} \xrightarrow{hv} K_{590} \Leftrightarrow L_{550} \Leftrightarrow M^1_{410} \Leftrightarrow M^2_{410} \Leftrightarrow N_{560} \Leftrightarrow O_{610} \rightarrow bR_{568}$$

([32]; reviewed by [1,2,33–37]). At least 11.6 kcal/mol is stored in the bR→K photoreaction [36] and is used to drive the subsequent thermal reactions of the photocycle. These reactions are coupled to the transmembrane translocation of a proton in bR that takes place in at least five distinct steps (Fig. 1). Two of these steps are proton release to the extracellular surface and proton uptake from the cytoplasmic surface (shown with dark arrows in Fig. 1). The other three steps are intramolecular proton translocations between buried residues, the Schiff base (SB), Asp85, Asp96 and the proton release complex (PRC).

2.1. Key groups involved in proton transport

The key functional element in bacteriorhodopsin is the chromophore, all-*trans* retinal, buried inside the protein and attached to lysine (Lys216) via a C=N bond called the Schiff base [38]. In the initial state of the pigment, bR_{568}, the Schiff base is protonated ($C=NH^+$). Light causes deprotonation of the Schiff base followed by proton release to the extracellular surface. Reprotonation of the Schiff base occurs from the cytoplasmic side and is coupled to proton uptake from that side [39]. This results in a transmembrane proton translocation. The bacteriorhodopsin often is schematically represented as consisting of two half-channels, a narrow cytoplasmic one and a wider extracellular one, connecting the Schiff base to both surfaces [20,40]. In the initial state, the Schiff base of the all-*trans* chromophore maintains connectivity to the extracellular channel [41–43].

Besides the Schiff base, other groups are involved in the light-induced proton transport and undergo reversible protonation–deprotonation reactions. Asp85 is particularly important. This residue is located at about 4.2 Å from the Schiff base and closer to the extracellular side [27,28]. It is negatively charged and serves as the primary proton acceptor when the Schiff base deprotonates [44,45]. Protonation of Asp85 at low pH or mutation of Asp85 to a neutral nonionizable residue inhibits or strongly reduces light-induced proton transport [46,47]. Asp85 can be titrated from the extracellular channel in the initial bR [42,48].

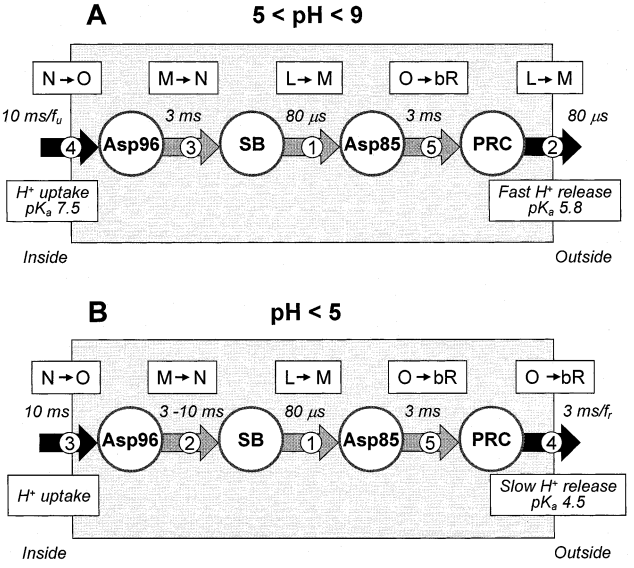


Fig. 1. Scheme showing steps in light-driven proton transport by bacteriorhodopsin from the inside the cell to the outside. Numbers at the arrows indicate steps of proton transport. Dark arrows stand for proton release and proton uptake to and from the bulk. Gray arrows are intramolecular proton transfers between buried groups. (A) Sequence of transfers at $5 < pH < 9$. 1, Transfer of a proton from the Schiff base (SB) to Asp85 during the L to M transition. 2, Approximately simultaneous proton release to the extracellular surface from the proton release complex, PRC (Arg82, Glu204, Glu194 and bound water molecules are key elements of this complex). This fast proton release vanishes at low pH with pK_a 5.8 [65]. 3, Transfer of a proton from Asp96 to the Schiff base during the M to N transition. 4, Uptake of a proton from the cytoplasmic surface and reprotonation of Asp96 (pK_a 7.5) during the N to O transition [13,16]; The rate of the transition is pH-dependent, decreasing at high pH with pK_a 7.2–7.5 ($f_u = 1/(1+10^{pH-7.5})$ [13]. 5, Transfer of a proton from Asp85 to the proton release complex in the extracellular channel during the O to bR transition. (B) Sequence of proton transfers at $2 < pH < 5$. At low pH, the sequence of proton release and uptake is reversed so that proton uptake occurs during N→O transition followed by proton release during the O→bR transition [65,82]. The rate of the O→bR transition decreases at low pH with a pK_a of the PRC in O, 4.5 ($f_r = 1/(1+10^{4.5-pH})$ [13].

A second important residue is Asp96. It is located at about 11 Å from the Schiff base in the cytoplasmic domain (channel), approximately halfway to the cytoplasmic surface [27,28]. Asp96 is protonated in the initial state of bR. It undergoes transient deprotonation in the photocycle serving as an internal proton donor to the Schiff base [44,49–51]. Mutation of this residue dramatically slows the photocycle, particularly at neutral and high pH [52].

The Schiff base is involved in the internal proton transfers to and from the proton acceptor (Asp85) and donor (Asp96) groups, whereas Asp85 and Asp96 communicate with the extracellular and cytoplasmic surfaces, respectively. Transmembrane proton transport is driven by light energy stored in the primary light reaction, the all-*trans*→13-*cis* isomerization of the chromophore [33,36]. This reaction initiates a sequence of changes including changes of accessibility (or connectivity) of the Schif base to the extracellular and cytoplasmic surfaces [2,3,53,54] and changes of proton affinities (pK_as) of the main groups, the Schiff base, Asp85, Asp96 and the proton release complex [3,6,8,13, 16,55–59].

2.2. Steps of proton transport in bR

During the first step the proton is transferred from the protonated Schiff base to anionic Asp85 in the L→M transition (Fig. 1). In order for this to happen the initially high pK_a of the Schiff base (12.4–13 [60,61]), and the initially low pK_a of Asp85 (ca. 2 in high salt [62]) should change so that this proton transfer becomes possible. These changes are caused by the all-*trans*→13-*cis* light-induced isomerization of the chromophore in the primary light reaction BR→K that destabilizes the Schiff base/Asp85 ion pair by changing geometry of this pair [63,64] and disrupting hydrogen bonding with the water molecule [25,31] that stabilizes this pair in the ground state [24,27,28].

The second step of the proton transport is the release of a proton to the extracellular surface from the so-called proton release group [65], or complex (PRC) [11], which occurs in the pH range between 5 and 9 (see Fig. 1A). The proton is released to the extracellular surface of bR almost simultaneously with formation of the M intermediate with a time constant of about 80 μs [66–69]. The proton release complex includes Arg82, glutamic acid residues, Glu204 and Glu194 and internal hydrogen bound water molecules [7,11,12,25,31,70,71]. Upon formation of the M intermediate, the pK_a of the proton release complex decreases from ca. 9.5 ([8,72], see below) to 5.8 [65].

The third step is the reprotonation of the Schiff base from Asp96 as a result of internal proton transfer [44,49,51,73]. It occurs during the M→N transition [74], or more precisely during M_2→N transition, taking into account the substates of M, M_1 and M_2, differing by access of the Schiff base to the extracellular and cytoplasmic half-channels, respectively [35,37,43]. In the ground state the pK_a of Asp96 is very high, above 11 [75]. During the M→N transition connectivity between the Schiff base and Asp96 is established and the proton affinity of Asp96 decreases so that its proton is transferred to the Schiff base. In the wild type (WT), the reaction occurs with a time constant of about 3 ms. It can be observed up to very high pH (>11). This indicates that during the M→N transition the connectivity of Asp96 with the bulk remains low and its pK_a remains high (see below).

The fourth step involves reprotonation of Asp96 from the bulk [76,77] and so proton uptake [19] in the N→O transition. During this step the pK_a of Asp96 decreases to 7.2–7.5 [13,16,78] due to increased connectivity of Asp96 with the cytoplasmic surface. It occurs with the time constant of ca. 5–10 ms as one can conclude from the kinetics of proton uptake at pH <7. The rate constant of proton uptake and the rate of the N→O transition decrease upon increasing the pH with the pK_a of 7.2–7.5 [19] due to slowing of reprotonation of Asp96. Proton uptake is accompanied by reisomerization of the chromophore from 13-*cis* configuration in N to all-*trans* configuration in O [33].

Finally, the fifth and the last step in the photocycle is the internal proton transfer from Asp85 to the PRC [79,80], resetting the protonation states of the residues of bR to their initial states. The set of main reactions that are involved in the transmembrane proton transport can be written in the following way:

$$[t\text{−}SBH^+/H_2O/Asp85^-] + h\nu \rightarrow c\text{−}SB+ \quad Asp85H + H_2O \quad (1)$$

$$XH \rightarrow X^- + H^+_{release} \quad (2)$$

$$Asp96H + c\text{−}SB \rightarrow Asp96^- + c\text{−}SBH^+ \quad (3)$$

$$Asp96^- + H^+_{uptake} \rightarrow Asp96H \quad (4a)$$

$$c\text{−}SBH^+ \rightarrow t\text{−}SBH^+ \quad (4b)$$

$$t\text{−}SBH^+ + H_2O + Asp85H+ \quad X^- \rightarrow [t\text{−}SBH^+/H_2O/Asp85^-] + XH \quad (5)$$

Here *t* and *c* stand for the all-*trans* and 13-*cis* configuration of the chromophore attached to the Schiff base. X is the proton release complex. The brackets indicate the ion pair stabilized by water molecule(s) [24,27,31,81].

At low pH (pH < 5) proton release from the PRC does not follow M formation but occurs on a millisecond time scale at the very end of the photocycle, during the O → bR transition [65,82] (Fig. 1B). It is prerequisite for the deprotonation of Asp85 in the O → bR transition, which makes the rate constant of this transition pH-dependent: it slows at low pH with the pK_a of PRC (4.5 in 150 mM salt) [13].

3. Protonation reactions in the initial state of bR

3.1. pK_as of the key groups in the initial (ground) state of bR

3.1.1. Schiff base

The pK_a of the Schiff base in bR is very high, above 12.4 (pH at which the alkaline denaturation of bR starts to occur) [60,61]. It sets the upper pH limit for the function of bR as a proton pump. The neutralization of the negative charge on Asp85 by the D85N mutation results in a shift of the pK_a of the Schiff base to ca. 8.5 [56,83,84], a value that is closer to that of model Schiff base compounds (ca. 7). This indicates that high pK_a of the Schiff base in bR is largely due to the Schiff base–Asp85 interaction. This ion pair is stabilized by a water molecule between them (Wat402) and a water molecule that interacts with the carboxyl of Asp85 (Wat401) [27] and by other interactions within the complex counterion to the Schiff [85], which also includes Asp212, Arg82 and water molecules. In the M_2 intermediate where Asp85 is protonated and the Schiff base is accessible from the cytoplasmic channel, the pK_a of the Schiff base was determined for the D96N mutant [59], in which the Schiff base equilibrates with the bulk in the presence of azide [86]. The pK_a had approximately the same value (8.2–8.3) as in the initial state of the D85N mutant [59], indicating that protonation of Asp85 results in a decrease of the pK_a of the Schiff base by about 5 pK units. Mutation of negatively charged Asp212 did not cause a comparable reduction in the ground state pK_a of the Schiff base [87]; however, substitution of a positively charged Arg82 with a neutral residue in addition to the D85N mutation increased the pK_a of the Schiff base by 2.2 pK units [56].

3.1.2. Asp85

Asp85 is a part of a complex counterion to the Schiff base [85]. The pK_a of Asp85 is sensitive to salt concentration. At high salt (> 3 M KCl) the pK_a is close to 2 [62], and increases at low salt, which may be explained by screening with cations of the negative surface charges due to lipid [88,89] and carboxyl groups [62]. The negative surface charge causes an increase concentration of protons near the surface in the absence of cations. The protonation of Asp85 at low pH [45] is accompanied by a 35 nm red shift of absorption maximum caused by conversion of the initial purple membrane (568 nm) into the so-called blue membrane (603 nm) which does not pump protons. Thus in the wild-type the pK_a of Asp85 sets the low pH limit for the function of bR as a proton pump [90]. The pK_a of Asp85 is sensitive to the mutation of certain residues. Substitution of Arg82 with a neutral residue causes the largest shift of the pK_a of Asp85 from 2.6 to 7.2–7.5 [6,56,91]. The proton affinity of Asp85 is coupled to the protonation (and conformational) state of PRC [6,7] as discussed in a separate section below.

3.1.3. Asp212

Asp212 has a very low pK_a, below 1 [45]. The

ionized state is stabilized by strong hydrogen bonding of Asp212 to Tyr185, Tyr57 and two water molecules [27]. Electrostatic interactions with the positively charged Schiff base and Arg82 are likely to contribute to the very low pK_a of Asp212. A blue shift of the absorption spectrum from 603 to 584 nm with pK_as 1 (minor) and 0 (major) seen in wild-type bR was assigned to protonation of Asp212 [7]. This absorption shift is sensitive to substitution of Arg82 with a neutral residue, which supports the hypothesis that it may be caused by the protonation of Asp212: in R82A a similar blue shift takes place with pK_a 3 [7]. This transition occurs in the absence of Cl ions which are known to cause a blue shift and the formation of so-called acid purple species [92,93] presumably due to the chloride replacing Asp85 as the counterion to the Schiff base [94]. It was suggested that protonation of Asp212 is involved in the formation of acid purple species [95]. Asp212 is part of the complex counterion to the Schiff base [85,87]. Mutation of Asp212 strongly affect the photocycle and decrease proton pumping efficiency [87]. Asp212 may undergo transient protonation in the photocycle and may be involved in the proton transfer from Asp85 to the PRC [96].

3.1.4. Glutamic acid residues at the extracellular surface

Four glutamic residues are located close to the extracellular surface, Glu9, Glu194, Glu204, and Glu74. Two of them, Glu204 and Glu194, are part of the proton release complex [11,12,25]. Glu194 presumably has a pK_a below 3 [96]. However, estimates of the pK_as of individual amino acid groups in this strongly coupled domain are difficult and need further verification and reconciliation with the view that PRC could be the whole hydrogen bonded network including Arg82, Glu204, Glu194 and water molecules [11,71].

Substitution of all four glutamic acid residues with glutamine produced a pigment that was functional in 1 M KCl but had no capability for fast light-induced proton release. The pigment showed unusual sensitivity to the chloride anion in solution. In low salt and in 1 M Na_2SO_4 a large shift of the absorption maximum from 558 to 512 nm was observed [97]. That was interpreted as an indication that the negative charge due to some of these residues is important for maintaining the functional state of the pigment.

3.1.5. Asp96

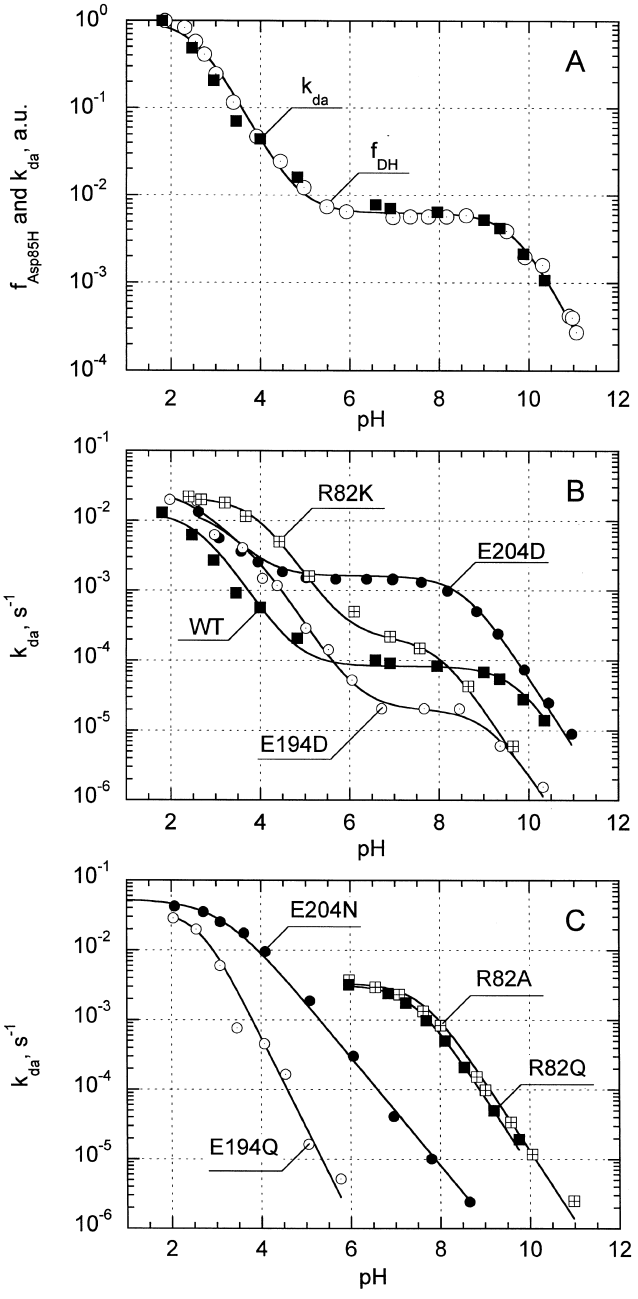
Asp96 is an internal proton donor to the Schiff base [44,49,50,98]. The pK_a of Asp96 in the initial state is above 11 [73,75,99]. It is surrounded by a very hydrophobic environment, consisting of Ile45 and several leucine residues, which make nonpolar barrel around Asp96 and two phenylalanines which can be viewed as part of the lids or gates, on the way to the Schiff base (Phe219), and to the cytoplasmic surface (Phe42) [25,27,28]. The only polar residue in the close vicinity of Asp96 is Thr46, which is hydrogen bonded to the protonated carboxyl group, stabilizing the protonated state. During the M $\Leftrightarrow$ N transition the proton affinity of Asp96 decreases [16], and its proton is transferred to the Schiff base. During the formation of the O intermediate reprotonation of Asp96 from the bulk takes place [77].

3.1.6. Carboxyl groups on the cytoplasmic surface

On the cytoplasmic surface there are four aspartic acid residues, Asp36, Asp38, Asp102 and Asp104, and in the EF loop are two glutamic acid residues, Glu161 and Glu166. Three glutamates (Glu232, Glu234 and Glu237) and one aspartate (Asp242) are located also on the C-terminus. These residues are presumably ionized at neutral pH [100]. It has been proposed that the negative charge produced by these residues on the cytoplasmic surface [62,101] might serve as an antenna for capturing protons from the bulk and concentrating them near the entrance to the cytoplasmic channel [102]. Single replacements of Glu161, Asp36 and Asp38 for cysteine decreased the rate of proton uptake by about twofold [103]. Mutation of Asp38 also slowed the rate of the preceding internal proton transfer from Asp96 to the Schiff base (the fast phase of M decay) by five- and threefold in the D38C [103] and D38N [104] mutants, respectively. At the same time the D38N mutation accelerated N decay at high pH [104].

In the wild-type bR, at low pH a threefold decrease in the rate of the M $\rightarrow$ N transition is observed with pK_a ca 4.5–5 [13,104]. It is possible that this process can be associated with the protonation of Asp38 at low pH, since mutant studies indicate that neutralization of negative charge on Asp38 de-

creases the rate of M decay. It has been suggested that Asp38 controls the rate of conformational change that causes transient decrease of the pK_a of Asp96 and reprotonation of the Schiff base [103]. The D38R mutation results in a 15-fold slowing of M decay and sevenfold slowing of proton uptake [103]. The triple mutation D36N/D38N/D102N reduced the rate of proton transport by only threefold at high light intensity [104], and among 11 variants of bR found in nature, only Asp36 and Glu166 are conserved [104]. Mutant studies indicated that these residues might be important for optimization of the proton transport but are not strictly essential.



3.2. Catalytic role of transient protonation of Asp85 in the thermal isomerization of the chromophore (dark adaptation) in bR

In the light adapted state the chromophore of bR has an all-*trans* configuration. In the dark a slow process of thermal isomerization around the 13C=14C [105] and 15C=N [106] bonds, called dark adaptation, takes place. At equilibrium, the mixture contains about 40% all-*trans*, 15-*anti* and 60% 13-*cis*, 15-*syn* chromophore. It has been known for a long time that the rate of this process is pH-dependent [107]: the rate constant does not change between pH 5 and 8 but it increases at lower pH in parallel with the purple-to-blue transition and decreases at high pH (Fig. 2A). It was suggested that these changes in the rate constant might be caused by changes in the protonation states of two or three groups [108]. The amino acid residue that is protonated during the purple-to-blue transition was shown to be Asp85 [45,91]. Comparison of the rate constant of thermal isomerization and the fraction of protonated Asp85 in R82A [6], R82K [7] and then in the WT [8] showed that the rate constant of thermal isomerization is proportional to the fraction of protonated Asp85 (or fraction of blue membrane): $k_{da}(pH) = k^{o}_{da} f_{Asp85H}$. This simple relationship indicates that thermal isomerization even at high pH

←

Fig. 2. Titration of Asp85 in wild-type bacteriorhodopsin and in Arg82, Glu194, and Glu204 mutants. (A) Comparison of the pH dependence of fraction of protonated Asp85 (f_{Asp85H}) and the pH dependence of the rate constant of dark adaptation, k_{da}, in wild-type bR. The latter was normalized to f_{Asp85H} at the maximum [8]. (B) pH Dependence of rate of thermal isomerization in WT, R82K [7], E194D, and E204D [13] mutants. The complex two component curves indicate coupling of the protonation states of Asp85 and the proton release complex as described by the model in Fig. 3. (C) pH Dependence of thermal isomerization in R82A(Q) [6], E194Q, and E204N [13] mutants. A single component pH dependency indicates elimination of coupling in these mutants. Similar results were obtained with the E204Q mutant [10].

occurs upon spontaneous protonation of Asp85 and transient formation of the blue membrane. The proportionality is observed over more than 3 orders of magnitude (upon decreasing the fraction of blue membrane from about 100% at low pH to 0.1% at high pH) for the WT and several mutants [6,8,109]. The rate constant of thermal isomerization is at least 5000 times faster when Asp85 is protonated than when it is deprotonated (in purple membrane). Thermal all-*trans* ⇔ 13-*cis* isomerization of the chromophore includes three steps: (i) transient protonation of Asp85 and formation of blue membrane; (ii) isomerization of the chromophore in the blue membrane; (iii) deprotonation of Asp85 [6,8]. At neutral pH, the fraction of protonated Asp85 is only about 0.7%, explaining the reduced rate of the chromophore thermal isomerization as compared with the rate at low pH. Protonation of Asp85 eliminates its electrostatic interaction with the protonated Schiff base decreasing the barrier for isomerization by about 4.5 kcal/mol [6]. This mechanism agrees with earlier predictions that the elimination of the negative charge of the counterion should result in the increased delocalization of the π-electrons in the chromophore and so decrease the order of the double bonds [110]. Theoretical calculations of the effect of protonation of Asp85 on thermal isomerization gave a value of 6 kcal/mol [15], close to the experimental value. In some mutants, E204Q [10] and R82H [14], a pH-independent basal rate of isomerization which extends to high pH, is observed. It was assigned to a rate constant of isomerization in the purple membrane (when Asp85 is deprotonated). In the R82H it was four orders of magnitude less than the rate constant in the blue membrane.

It is reasonable to expect that the catalytic effect of protonation of Asp85 on the chromophore's thermal isomerization, that was revealed in the dark adaptation experiments ([6,8], is likely to take place also in the photocycle, during the N to O transition, where the chromophore undergoes thermal reisomerization around 13C = 14C double bond [111]. Asp85 stays protonated (has a high pK_a) until the very end of the photocycle and so probably is involved in the catalysis of the chromophore reisomerization [8] which occurs during the N → O transition (perhaps actually between two substates of O intermediate [1]). Reisomerization of the chromophore in the N → O transition is facilitated also by protonation of Asp96 [13,112,113].

3.3. Complex titration curve for Asp85: evidence for coupling between Asp85 and PRC in the initial state of bR

A surprising feature was found upon titration of the purple-to-blue transition caused by protonation of Asp85. Besides the main well known transition with pK_a around 2.6 there was a second transition at high pH (with pK_a 9.5–9.7) [8], see Fig. 2A. In the WT this second transition is difficult to detect because only 0.7% of the pigment undergo this transition [8]. However, in some mutants, for instance in R82K [7], a larger fraction of the pigment titrates with a high pK_a. The complex titration of Asp85 was explained by assuming that the proton affinity of Asp85 (pK_a) depends on the protonation state of another residue (or complex of residues) termed X′. Deprotonation of this residue at high pH results in an increase of the pK_a of Asp85, and vice versa, protonation of Asp85 causes a decrease in the pK_a of this group. The size of the change in the pK_a of one group when the other changes its protonation state is called the coupling strength [13]. The model that describes this linkage is shown in Fig. 3. It provides a tool to estimate the pK_as of Asp85 and X′ from a fit of the experimental titration curve. This model proved to be useful in understanding several phenomena related to proton transfer reactions in the photocycle.

For the WT the titration curve for dark adaptation [6,8,107], and proportional to it the titration curve of the purple-to-blue transition [8], indicate that upon deprotonation of group X′ the proton affinity of Asp85 increases by almost 5 pK units, from 2.6 to 7.5. This explains the plateau on the titration curve between pH 5 and 9 (Fig. 2A). Conservative mutations R82K, E204D and E194D alter the titration of Asp85, determined from the pH dependence of dark adaptation (see Fig. 2B), but retain the two component shape of the titration curve. For example, the R82K mutation increases the pK_a of Asp85, decreases the pK_a of X′ and reduces the coupling strength between Asp85 and X′ by half [7] which results in a closer position of the two components. The E204D mutation increases whereas the E194D

mutation decreases the fraction of protonated Asp85 and the rate of dark adaptation at neutral pH (Fig. 2B), affecting the position of the plateau on the titration curve.

Balashov et al. [6] found that the R82A mutation eliminates the complex titration of Asp85 and the coupling of Asp85 with the group X′ (Fig. 2C). A similar feature is exhibited by the R82Q [10], E204Q/N [10,13] and E194C/Q mutants [11–13]. This showed that Arg82, Glu204 and Glu194 are key parts of complex X′ deprotonating at high pH with pK_a 9.5, or parts of a linkage that couples the proton affinity of Asp85 with the protonation state of X′.

3.3.1. *The nature of the group X′ affecting the pK_a of Asp85: its relationship with the PRC*

The dramatic increase of the pK_a of Asp85 by about 4.6 units caused by R82A and R82Q mutations lead to the suggestion that the increase of the pK_a of Asp85 at high pH is due to: (i) deprotonation of Arg82 or a water molecule associated with Arg82 or (ii) the motion of a side chain of Arg82 away from Asp85 at high pH caused by the deprotonation of some group on the surface [6]. Estimations of the pK_a of Arg82 using the titration of different groups gave a high value, about 13.8 [56] excluding Arg82 as being the group deprotonating with pK_a 9.5. On the other hand, motion of Arg82 in response to deprotonation of some surface group is a likely possibility [6,114,115]. Recently the displacement of Arg82 by about 1.6 Å towards extracellular surface was indeed observed upon formation of the M like intermediate in the D96N mutant [31].

The nature of the linkage between Asp85 and X′ makes X′ an ideal candidate for the PRC [6–8,10]. Upon protonation of Asp85 the pK_a of X′ drops to 4.8 (Fig. 3) which would lead X′ to release a proton if the external solution were at pH 7. The pK_a of X′ of 4.8 is somewhat below the pK_a of the proton release group in M of 5.8 [65]. The difference of one pK_a unit was suggested to be caused by a different protonation state of the Schiff base in M (unprotonated) as compared with the blue membrane (protonated) and to the different chromophore configuration (13-*cis* in M and mostly all-*trans* in BM) [6,7].

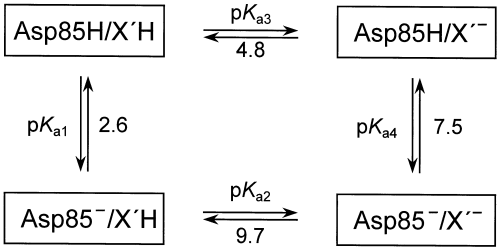


Fig. 3. Scheme describing coupling of the protonation of Asp85 with the protonation state of another residue X′, to explain the complex titration curve of Asp85 [6–8]. The fraction of protonated Asp85 (blue membrane) is given by a function of three pK_as: $f_{Asp85H}(pH) = \alpha/(\alpha+\beta\gamma)$, where $\alpha = 1+10^{(pH-pKa3)}$; $\beta = 1+10^{(pH-pKa2)}$; $\gamma = 10^{(pH-pKa1)}$. This model can be applied to different pairs of residues (for instance Asp85 and Asp212) and expanded to a larger number of interacting residues [14].

Further evidence for X′ and PRC being the same entity came from mutant studies. The R82A and R82Q mutations abolished both fast proton release [6,70,83] and coupling of X′ with Asp85 [6]. Similar elimination of fast proton release and coupling of Asp85 and proton release group (as indicated by the lack of the high pK_a transition in the pH dependence of dark adaptation) was found for the E204Q mutant [10,70]. The subsequent finding that the mutation of another residue, Glu194, also eliminates fast proton release and complex titration of Asp85 [11] strongly suggested that the proton release group (and so X′) is not a single residue but rather is a complex of residues (PRC) including Arg82, Glu204 and Glu194 and water molecules [11,12]. The latter pair of residues is close to the extracellular surface and can be viewed as the terminal part of the chain of residues including Arg82 and water molecules that connect the Asp85–Schiff base region with the extracellular surface [101]. Dioumaev et al. [12] presented evidence that Asp194 in the unphotolyzed E194D pigment is deprotonated and has a pK_a below 3, and so presumably works as a terminal proton release group. The role of Glu204 and the nature of the group that deprotonates in the ground state with pK_a 9.7 still need further investigation. It was proposed that it is a complex [11] or a network of several extracellular residues and water molecules [71], or a water molecule [24] or hydronium ion [26] associated with Arg82 and Glu204, or the Glu204–Glu194 pair [25].

3.3.2. *Implications of coupling between the PRC and Asp85 for the photocycle reactions; role of the pK_a of Asp85*

An important implication follows from the existence of coupling between Asp85 and PRC: protonation of Asp85 facilitates deprotonation of PRC. The opposite relationship is also true: deprotonation of PRC results in the increase of the pK_a of Asp85 and thus facilitates deprotonation of the Schiff base in the photocycle. It is known that at high pH the light-induced deprotonation of the Schiff base occurs about 100 times faster than at neutral pH [90,116,117]. The pK_a of this transition is about 9.2 in 150 mM KCl [61]. The 85-μs photocurrent caused by the proton movement from the Schiff base to Asp85 and the release of the proton to the surface, disappears with the same pK_a [72,90]. The total area under current associated with M formation is reduced by half at high pH [90]. These data can be interpreted as consequences of the deprotonation of the proton release group in the unphotolyzed state of bR and thus the elimination of fast proton release at high pH [8,72]. For the E204D mutant, which has a decreased pK_a of the proton release group, it was shown that slow proton release takes place at high pH [12].

The speeding up of the rate of the proton transfer from the Schiff base to Asp85, was also attributed to the deprotonation of the PRG at high pH, which would induce an increase in the pK_a of Asp85 according to the coupling model [7], and a change of the environment of the Schiff base/Asp85 ion pair leading to a 1.5 nm red shifted 'alkaline form' of bR [61]. This alkaline bR has its own photocycle (Fig. 4) with a twofold longer lifetime of the excited state [118], has no fast proton release [72], but has an increased rate of M formation [61,90] (Table 1). This increase in the rate is absent in the E204Q [70] and E194C mutants [11], which provides further evidence that the increase observed in the WT is associated with the deprotonation of the PRC in the initial state. The electrogenic component associated with the formation of M is about two times less in E204Q than in the WT [119,120]. This agrees with the conclusion that proton release from the PRC is electrogenic and that it vanishes at high pH [66,72,90].

In R82A, R82Q and R82K the rate constant of Schiff base deprotonation is very fast (ca. 1 μs) as in the WT at high pH and does not show any substantial pH dependence [6,7]. This can be explained by a high pK_a of Asp85 in these pigments [109].

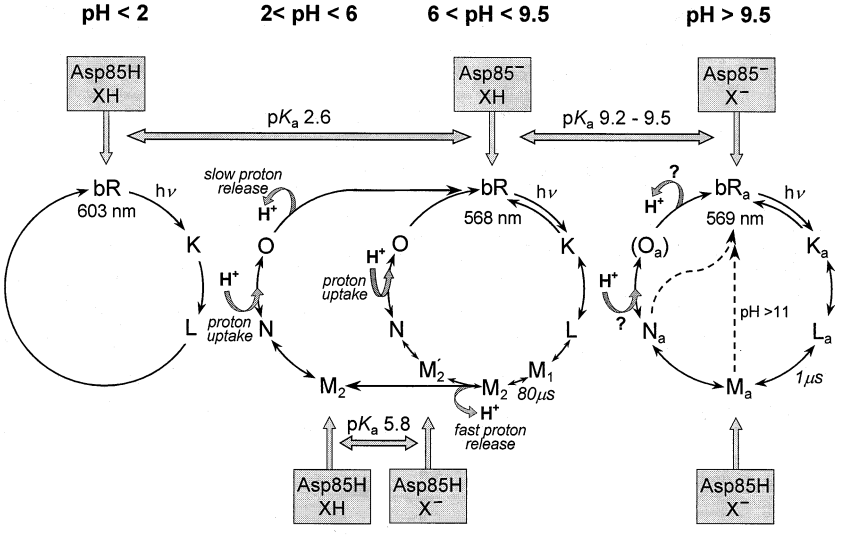


Fig. 4. Scheme of the photochemical conversion of bR at different pHs. At very low pH (pH < pK_a of Asp85), Asp85 is protonated and no M is formed. At high pH, the proton release complex (denoted X) is deprotonated, and the alkaline form of bR is formed, bR_a. This species has a slightly red-shifted absorption spectrum [61] and very fast M formation, but proton release is abolished [72]. At a pH close to the pK_a of the PRC in M (5.8), branching of the photocycle occurs [35]. At a pH less than the pK_a of PRC (X), proton release takes place at the end of the photocycle during the O → bR transition [65]. The substates on the N intermediate and O intermediate discussed in the text are not shown in this simplified scheme.

Table 1
pH Dependence of the rate constants and yields of photocycle reactions in bR

Description of reaction[a]	pK_a[b]	Likely cause	Ref.
Twofold increase in the lifetime of the excited state and decrease of the rate pISO[c]	≈9.0	Change of the chromophore environment (due to deprotonation of PRC)	[118]
Increase in the fraction of fast component of M formation	9.2	Deprotonation of PRC in bR	[61,116]
Decrease of the photocurrent associated with fast H^+ release	9.2	Deprotonation of PRC in bR	[72]
Shift of the L⇔M equilibrium towards M	5.8	Deprotonation of PRC in M	[65]
Increase in the fraction of fast proton release	5.8	Inability of PRC to deprotonate at low pH in M	[65]
Threefold decrease in the rate of the M→N transition	4.5	Protonation of Asp38	[13,104]
Decrease in the rate of N→O transition and of the fraction of O at high pH [13,16,17,151,154]	7.2–7.5	Slowing of reprotonation of Asp96	
Decrease in the rate of proton uptake	7.2–7.5	Slowing of reprotonation of Asp96 at high pH	[19]
Decrease in the rate of proton uptake	11.5	Slowing of reprotonation of Asp96 in its high pK_a state	[148]
Increase in the rate constant of the O→bR transition and decrease in the fraction of O	4.5	Slowing of deprotonation of Asp85 at low pH because PRC remains protonated	[13]

[a]Effects are described for increasing pH.
[b]pK_a values are given for 100–150 mM salt.

Further evidence that the pK_a of Asp85 and interaction of Asp85 with Arg82 play key roles in the light-induced proton transport was obtained with the R82H mutant. This mutant shows an unusually low pK_a of Asp85 (ca. 1) [14]. At pH 3 the pigment is purple but no M is formed, indicating that the low proton affinity of Asp85 in the unphotolyzed state persists in the L⇔M transition. The amount of M and the rate constant of its formation, however, increase with pK_a 4.5 and 9 upon increasing the pH, indicating that deprotonation of two groups increase the proton affinity of Asp85. The group with pK_a 9 is most likely the same group that causes increase in the rate of M formation in the WT (X′ or PRC). The low pK_a (4.5) group was tentatively assigned to Glu194 or Asp212 [14]. Thus the high pK_a proton release group is not the only group which is coupled to Asp85 in bR and is able modulate its pK_a.

3.3.3. *Mechanism of coupling between Asp85 and the proton release complex*

Asp85 is located at more than 12 Å from the extracellular surface. The question is how the protonation state of Asp85 is coupled to the protonation state of the PRC. There are several observations showing that the kinetics of proton release do not follow exactly the kinetics of M formation in the WT and mutants [69]. Proton release is substantially delayed compared with M formation in the D85E [121] and E194D [12] mutants. This suggests that a conformational transition in the protein is involved in the transmission of the protonation event from Asp85 to PRC. An earlier analysis also suggested that a protein conformational transition is the rate limiting step in M formation [122]. Recent structural studies by Luecke et al. [31] indicate that upon M formation, the environment of Asp85 becomes less polar and the Arg82 side chain and hydrogen bonded water molecules move towards the extracellular surface. It is likely that this movement of Arg82 and the waters causes the decrease in the pK_a of the PRC [6,31,115,123]. This movement also results in an increase of the pK_a of Asp85 [6] and so facilitates proton transport from the Schiff base to Asp85. It is presumably the key part of the coupling mechanism [6,31] and part of the switch that disconnects the Schiff base with the extracellular side and connects it to the cytoplasmic side [10,31,43].

It is likely that in the unphotolyzed state of bR the reverse sequence of events takes place: deprotonation of the proton release group at high pH shifts the equilibrium between two possible positions of

Arg82 toward the one closer to the extracellular side [6,114,115]. This causes an increase in the pK_a of Asp85 by about 5 pK units due to both changes in electrostatic interactions [6] and in hydrogen bonding with polar residues and water molecules [7,14,31,124]. A small red shift of the chromophore and tryptophan absorption bands seen in the WT [61] and some mutants such as R82K [7], and R82H [14], but not in R82A, E204Q or E194Q, indicates changes in the chromophore environment at high pH.

4. Proton transfer reactions in the photocycle

4.1. Mechanism of light-induced deprotonation of the Schiff base and proton release

After photoisomerization of the chromophore during the bR→K transition [29,33,36] and subsequent relaxation of K into L, the Schiff base/Asp85 ion pair is perturbed. The N–H bond of the Schiff base, initially pointed to Asp85 [27,125], must be turned away from Asp85 as a consequence of photoisomerization. The water molecules that are hydrogen-bonded to the Schiff base and to Asp85 in initial bR exhibit shifts in frequencies of their O–H vibrations, indicating stronger hydrogen bonding in L [81,126]. Solid state NMR data indicate a stronger interaction of the protonated counterion and the Schiff base in L [127]. These changes in geometry and hydrogen bonding are the likely cause of the change in the proton affinities of the Schiff base and Asp85 that lead to the proton transfer [63,64]. The transfer of a proton from the Schiff base to Asp85 in the L to M transition is likely to involve one or more water molecules that stabilize the Schiff base and Asp85 in L [31,128–130] and formation of several substates of M [23,35,131–135].

The exact mechanism of proton transport from the Schiff base to Asp85 in the L→M transition is yet unknown. One of the possible scenarios is that the water molecule between the Schiff base and Asp85 (Wat402), which receives a hydrogen bond from the Schiff base and donates hydrogen bonds to Asp85 and Asp212 in the initial bR [27,28], is strongly polarized by the Schiff base and Asp85 in L. This facilitates the water's dissociation [27] with it donating a proton to Asp85 and its hydroxyl to the Schiff base (to form a new water), thus neutralizing both groups. An alternative possibility is that the Schiff base dissociates giving up its proton to water 402 (or other water that interact with the Schiff base in L) that then donates another proton to Asp85. The first mechanism would be similar to interaction of negatively charged chloride ion with the protonated Schiff base as in the photocycle of halorhodopsin, a pigment closely related to bR [4].

These models are based on the assumption that Wat402, which bridges the Schiff base and Asp85 in the initial bR, is still there in the L intermediate. This, however, is not clear. The X-ray crystal structure of L is not available yet. Wat402 has not been detected in the X-ray crystal structure of M [31]. During the bR→K photoreaction the hydrogen bonds of Wat402 with the Schiff base and Asp85 are presumably disrupted [29]. This change of geometry and hydrogen bonding might be a key step that causes the destabilization of the Schiff base/Asp85 ion pair and leads to the proton transfer from the Schiff base to Asp85. A valuable source of information on participation of internal water molecules in the photocycle is light-induced changes of water vibrational bands observed in Fourier transform infrared (FTIR) difference spectra [81,136,137]. Photoexcitation of the L intermediate showed that light-induced isomerization of the chromophore in L causes a perturbation of at least two water molecules, one is in the vicinity of Asp85 [129]. These waters can participate in the stabilization of the ion pair in L and in the proton transfer from the Schiff base to Asp85 during the L→M transition. An FTIR study of the photoconversion of the M intermediate showed that two–three water molecules undergo perturbation upon photoisomerization of the chromophore in the M→M′ photoreaction at 80 K [138]. One is located close to Phe219.

The kinetics of M formation (Schiff base deprotonation) includes 2–3 components at neutral pH [65,69,121,139], which exhibit different deuterium isotope effects [130]. During the first (2–6 μs) phase only a small fraction of the Schiff base is deprotonated. This is followed by two subsequent phases which probably reflect conformational changes which accompany formation of the M intermediate [31].

These changes result in a shift of the equilibrium from an ionized pair (protonated Schiff base/ $Asp85^-$) in L to a neutral state (Schiff base/ Asp85H) in M. One of these conformational changes is associated with the proton release to the extracellular surface. The L⇔M equilibrium is more strongly shifted towards M at pH 7 than at pH 5, where no fast proton release is observed [65]. Qualitatively this shift can be explained by the existence of coupling between the pK_a of Asp85 and the protonation state of the proton release complex [6–8,10]: deprotonation of this complex raises the pK_a of Asp85 and thus stabilizes the protonation state of Asp85. The shift in the pK of the L⇔M equilibrium associated with the deprotonation of PRC per se is not very large, ca. 0.5–1 pK units [65,140]. This indicates that other intramolecular conformational changes are the cause of the major change in the proton affinity of Asp85 and the Schiff base. It was suggested that an increase in the pK_a of Asp85 might be caused by movement of Arg82 away from Asp85 [6,115,123] and involve changes of hydrogen bonding of groups and water molecules that interact with Arg82 [7,14].

As seen in the structure of the M-like intermediate of the D96N mutant [31], the Schiff base is displaced in a more hydrophobic environment. Two major changes occur in the extracellular domain upon Schiff base deprotonation: rearrangement of internal water molecules and the movement of Arg82 away from Asp85. Both changes create a less polar environment around the Schiff base and Asp85 that has been detected earlier in an FTIR study [58]. The water molecule 402 that stabilizes the Schiff base is not seen in M [31]. The protonated state of Asp85 is stabilized by a hydrogen bond to the oxygen of Wat401. The side chain of Arg82 is moved down about 1.6 Å from Asp85 to the extracellular surface. This movement helps to stabilize the neutral state of the ion pair (the M intermediate).

As a consequence of Asp85's now less polar environment, of its absence of interaction with the Schiff base, and of its reduced interaction with Arg82, the pK_a of Asp85 is increased to >11 [58]. This increase in the proton affinity of Asp85, produced by the rearrangement of bound waters, the displacement of the Arg82 side chain and the deprotonation of PRC, makes Asp85 a poor proton donor to the Schiff base and decreases the rate of reverse proton transfer reactions [65,141].

The photoexcitation of M and the subsequent chromophore photoisomerization to all-*trans* results in fast reversed proton transfer from Asp85 back to the Schiff base, restoration of the Schiff base/Asp85 ion pair and completion of the photocycle through a nonpumping pathway ([142]; for review see [109]), indicating that changes in the environment of Asp85 can be restored not only upon completion of the photocycle but also by a short cut reaction initiated by the photoisomerization of the chromophore back to its all-*trans* configuration.

Elimination of the electrostatic interaction between the Schiff base to Asp85, as a consequence of the light-induced proton transfer between these two key groups, induces a conformational change in the pigment [23,143,144] which propagate to the surface and involve motion of loops [145]. Certain features of this change persist through the N and O intermediates [30] and are reversed only upon deprotonation of Asp85 and the restoration of the Schiff base/ Asp85 ion pair. This provides a structural basis for viewing the functioning of bR and related sensory receptors as shuttling between these two major conformations [146,147].

4.2. *Mechanism of reprotonation of the Schiff base and proton uptake*

During the M→N transition a proton is transferred from Asp96 to the Schiff base. Within 2–3 ms an equilibrium with approximately equal concentrations of M and N is reached [148]. The reaction is almost pH-independent and is observed up to very high pH ca. 11 [73,111,149].

The next step is reprotonation of Asp96 which occurs during the N to O transition [77,150]. The rate constant of the N→O transition is pH-dependent with pK_a ca. 7.2–7.5 in the WT [13,151]. This suggests that the pK_a of Asp96 during its reprotonation in the N→O transition is about 7.2–7.5 [13] and that the decrease in the fraction of the O intermediate at high pH [152–154] is caused by a slowing of proton uptake and the reprotonation of Asp96 [13,19]. FTIR measurements of the amplitude of the 1740 cm^{-1} COOH band of protonated Asp96 provided a similar value for the pK_a of Asp96 in N

[16]. The same pK_a for the rate constant of the N→O transition was observed in the mutants with a disabled PRC (E204Q, E194Q) [13] which excludes participation of the proton release complex in the control of the N→O transition with pK_a 7.2–7.5. These data indicate that in N a transient lowering of the pK_a of Asp96 to ca. 7.2 (from >11 in initial bR) takes place. The light-induced decrease in the pK_a of Asp96 to ca. 7 and the unusual light-induced proton release from Asp96 was observed in the N intermediate of the D212N mutant [78] in which the Schiff base remained protonated during the photocycle (no M intermediate was formed). This indicates that transient decrease of the pK_a of Asp96 is not triggered by the Schiff base deprotonation but perhaps by the chromophore isomerization in the bR→K photoreaction [5].

This raises a question: if the pK_a of Asp96 decreases to 7.2 during the M→N transition how can it serve as a proton donor to reprotonate the Schiff base at high pH (pH>9)? Why does Asp96 not lose its proton to the bulk? If one suggests that the pK_a of Asp96 is lowered to ca. 7.2–7.5 and that it deprotonates into the cytoplasmic half-channel and that this proton can go in both directions, to the Schiff base and to the bulk, then one should assume that the Schiff base is a better trap for the proton from Asp96 than the bulk. However, there are two observations that do not easily fit into this model. The first one is that the concentrations of M and N are approximately equal in the quasiequilibrium M⇔N at pH 10 [148]. This implies that the equilibrium constant K_i in the reaction of the internal proton transport between Asp96 and the Schiff base (Asp96H+SB⇔Asp96$^-$+SBH$^+$) is close to 1 and pK_i is close to 0 (pK_i = −log [M]/[N]). This is much smaller than needed to explain the observation that reprotonation of the Schiff base occurs at pH>10: assuming that the Schiff base titrates through the cytoplasmic (uptake) channel in N [59], and that the pK_a of the Schiff base can be estimated as a sum of the pK_a of Asp96 and pK_i (pK_a(SB)= pK$_i$+pK_a (Asp96)) [13], the pK_i must be about 4 in order to obtain the pK_a 11 for the Schiff base. This is 3–4 pK units higher than the experimental value of pK_i which is about 0.5. The second observation is that in the D96N mutant, the pK_a of the Schiff base in N was estimated to be 8.2 (not 10 or 11) [59]. In the D85N/D96N double mutant which mimics the protonated state of Asp85 in M and N, the pK_a of the Schiff base has a similar low value 8.3–8.6 in the initial state [41,155]. It is likely that the Schiff base in the WT has a proton affinity in the M intermediate similar to that as in these mutants because mutation of Asp96 produces only a local effect and does not change environment of the Schiff base [31]. If this is the case, then the presence of Asp96 in the WT should prevent equilibration of the Schiff base with the bulk during the M→N transition and effectively raise the pK_a of the Schiff base above 11. This implies that during reprotonation of the Schiff base Asp96 itself should not be in a fast equilibrium with the bulk (should have a pK_a>11); however in the next step, during the reprotonation of Asp96, the accessibility of Asp96 to the bulk should increase and that to the Schiff base decrease. There must be a switch of accessibility of Asp96 between these two directions [19,156] in addition to the transient decrease in the proton affinity of Asp96 in N [78].

This leads us to a model involving two (or more) N intermediates that differ by the connectivity of Asp96 to the bulk and to the Schiff base. Ames and Mathies [151] introduced two N forms, suggesting that reprotonation of the Schiff base occurs during the M→N$_1$ transition and proton uptake by Asp96 takes place in the N$_1$→N$_2$ transition. Further evidence for two N forms was provided by Zimanyi et al. [148] who found two kinetically different N forms, and that proton uptake occurs during the N$_1$→N$_2$ transition at high pH. The evidence for two N or more N forms were presented also in other studies [157–159]. The substates of the N intermediate might be helpful in understanding the pH dependence of the Schiff base reprotonation and the mechanism of proton uptake. We suggest that due to a structural change in the protein the proton affinity of Asp96 is reduced, and connectivity between Asp96 and the Schiff base is increased in the M→N$_1$ transition. This transition presumably involves changes of the Asp96 environment, water molecules and conformational changes of Phe219 that controls the connection between Asp96 and the Schiff base [31]. The connection of Asp96 with the bulk should be blocked at that moment. In the next step, N$_1$→N$_2$, the connectivity of Asp96 with the Schiff base decreases and

connectivity to the bulk increases. That may occur for instance by a flip of Asp96 carboxyl group after its deprotonation from an orientation facing the Schiff base to one facing the cytoplasmic surface or by relocation of mobile water molecules. After this switch of connectivity of Asp96 in the uptake channel, reprotonation of Asp96 from the bulk takes place. Since the pK_a of Asp96 is 7.2–7.5 at this moment of the photocycle [13,16], the expected pK_a for proton uptake should have a similar value. Recent measurements by Balashov et al. [19] are in agreement with this pK_a value. In the E204A mutant and in the WT the pH dependence of the rate constant of proton uptake is about 7.5. This is much lower than the previously reported value of 11.5, derived from the pH dependence of the estimated rate constants of the $N_1 \Leftrightarrow N_2$ transitions at high pH [148]. These two different estimates for the pK_a of proton uptake (7.5 and 11.5) may not necessarily contradict each other. It is possible that there are two kinds of proton uptake. The first one is faster at neutral pH. It operates through the relatively 'open' uptake channel and is characterized by the pK_a of Asp96 being 7.5. The second type of proton uptake is through the 'closed' channel, where the pK_a of Asp96 is about 11.5. The existence of these two types of proton uptake with different pK_as can provide an explanation for the pH dependence of the rate constant of recovery of bR at high pH [160] which has a slope substantially less than one (0.4), indicating that reformation of initial pigment is controlled by more than one protonation reaction.

The molecular mechanism for reducing the pK_a of Asp96 that leads to transferring its proton to the Schiff base and subsequent reprotonation from the bulk remain obscure. Mutant studies indicate that the breaking of hydrogen bond of Asp96 with Thr46 [148,161] might contribute to the decrease in the pK_a of Asp96. The increased hydration of Asp96 with water molecules facilitates the $M \rightarrow N$ transition as indicated by the inhibition of N formation by dehydration [162] even under such mild conditions as 66% glycerol [149]. Water molecules are present in the Asp96–Schiff base region [27,28,138,163,164]. These waters have some mobility in the cytoplasmic channel and can serve as a mobile proton carrier between Asp96 and the Schiff base [31,129,138,165]. The formation of N is accompanied by changes in the FTIR spectra, indicating a conformational change involving one or more peptide groups [73,99,150,166]. Evidence was obtained that in N, the protonated Schiff base might be hydrogen bonded through a water molecule to Val49 [164], whereas Asp212 presumably serves as a counterion [80,167]. Structural studies indicate outward tilt of cytoplasmic part of helix F [30,144,168–170]. This tilt most likely is related to the changes of proton affinity of Asp96 and provide an opening in the cytoplasmic channel for an increased hydration of Asp96 [171]. Time resolved measurements with spin labels [172,173] detected structural changes of the EF loop that correlate with the formation of N (Asp96 deprotonation) and N decay (Asp96 reprotonation). These changes might be related to the movement of helix F and be involved in deprotonation and reprotonation of Asp96.

4.2.1. Relationship of reprotonation of Asp96 and proton uptake with the chromophore's thermal reisomerization in the N → O transition

Studies of connection of proton uptake with the photocycle reactions in the WT [148,154] show a somewhat complicated relationship: at low pH proton uptake lags behind the formation of O and was suggested to occur in the transition between the two O states [154], whereas at high pH proton uptake follows the decay of N_1 and occurs presumably between the two N states [148]. However, this kinetic correlation of proton uptake with different reactions at low and high pH might only be apparent. There is substantial evidence that in WT bR, the $N \rightarrow O$ transition is three- to fivefold slower than the $O \rightarrow bR$ transition at pH 6–7 [13,77,139,151]. Studies of the pH dependence of the fraction of the O intermediate and the pH dependence of its formation and decay showed that in the WT the apparent rate constant of the formation of the O intermediate reflects the faster rate constant of the $O \rightarrow bR$ transition at neutral and moderately low pH rather than the slower rate constant of the $N \rightarrow O$ transition [13]. So the faster rise of the O intermediate (compared with kinetics of proton uptake) does not necessarily mean that the formation of the O intermediate (and chromophore isomerization) precedes proton uptake. The observed pH dependencies of the rate constants of the $N \rightarrow O$ transition can be explained by assuming that repro-

tonation of Asp96 with pK_a 7.2–7.5 and the accompanying proton uptake are the rate limiting steps for this transition [13] and hence for the thermal reisomerization of the chromophore.

In mutants with a defective proton release group and so a slowed O→bR transition, like R82Q, E204Q [70,112,174] and E194C [11], proton uptake coincides with the rise of the O intermediate and proton release coincides with O decay. In these mutants the experimentally observed rate constant of O rise is close to the molecular rate constant of the N→O transition [13]. At high pH, the slowing in the proton uptake and the slowing of the N→O transition (formation of O) are observed with the same pK_a of 7.5 [13,19]. These data strongly suggest that reprotonation of Asp96 is a prerequisite step for the chromophore 13 *cis*→all-*trans* isomerization in the N→O transition, and that protonation of Asp96 facilitates isomerization [11,112]. It is possible that protonation of Asp96 results in a disruption of hydrogen bonding of the Schiff base with water molecule(s) in the cytoplasmic domain described for the N intermediate [164], thus facilitating isomerization.

The conclusion on the role of protonation of Asp96 in the 13-*cis*, 15 *anti*→all-*trans* chromophore reisomerization in the photocycle finds support in experiments with the D85N mutant and related double mutants. In the initial (unphotolyzed) state of this mutant the pH-dependent transitions are observed between species that resemble the M, N and O intermediates of the photocycle of bR [84]. The N-like species has the 13-*cis*, 15-*anti* configuration of the chromophore in the D85N/F42C mutant. Asp96 is deprotonated in this state [113]. Protonation of Asp96 at lower pH (with the pK_a close to 8) correlates with the transition of the N-like species into the O-like species which contains a mixture of all-*trans* and 13-*cis*, 15-*syn* chromophores, thus providing evidence that protonation of Asp96 shifts isomeric equilibrium toward the all-*trans* isomer [113].

4.3. Deprotonation of Asp85 and recovery of the initial pigment: proton release from the PRC is the rate determining factor at low pH

At neutral pH proton transfer from Asp85 to the PRC in the O→BR transition [79,80] is faster than the preceding proton uptake and chromophore isomerization in the N→O transition [13,139,151]. The deprotonation of Asp85 presumably should involve the movement of Arg82 closer to Asp85 and the restoration of Schiff base/Asp85 ion pair in a highly polarizable environment. This transition involves reprotonation of the PRC. The pathway for the proton is not known but one may speculate that a chain of hydrogen bonds that extends from Asp85 to Asp212 (through water 401), Tyr57, and Arg82 to Glu194–Glu204 site might be involved in this proton transport step. Evidence was presented that transient protonation of Asp212 might be involved in Asp85 deprotonation [96]. Mutation of E204 (E204Q) [70,112,175], Glu194 (E194C/Q) [11,13,96] and also of Tyr57 [2] and Thr205 [176] greatly slows the O→BR transition and results in the accumulation of large amount of O, indicating that deprotonation of Asp85 is greatly slowed by either alteration of a pathway (mutations of Tyr57, Thr205) or the lack of the proton acceptor in the E204Q and E194Q mutants.

In the wild-type protein, upon lowering the pH the fraction of the O intermediate increases at pH below 5 whereas the rate constant of the O→bR transition decreases [13,17] with pK_a 4.5, which is close to the pK_a of the proton release group in the blue membrane [8]. This indicates that the proton release group might be controlling the rate of deprotonation of Asp85 and the recovery of bR in the WT at low pH. The most plausible interpretation is that the proton release complex is the terminal acceptor of a proton from Asp85 not only at neutral but also at low pH [13]. Deprotonation of Asp85 can occur only after deprotonation of PRC. Bypass is possible but the rate of deprotonation of Asp85 bypassing the PRC is very small [13]. Thus at low pH as at neutral pH, deprotonation of Asp85 occurs mainly through the proton release complex. Further support for this conclusion was obtained with mutants that affect the pK_a of the PRC. In the E194D mutant this pK_a is increased both in M and in the blue membrane by about 1 unit. This correlated with the increase in the pK_a of the rate constant of the O→bR transition [13]. A similar effect is found for another mutant, R134H [18]. From these measurements the pK_a of the proton release group in O was estimated. It is about 1 unit lower than the pK_a of the PRC in M. In all these cases the rate constant for the recovery of

bR (the O→bR transition) is a function of the pK_a of the proton release complex which can be expressed with the simple equation $k_{O\rightarrow bR} = k_0/(1+10^{(pK-pH)})$, where k_0 is the rate at neutral pH [13]. In the mutants with a disabled PRC like E204Q and E194Q, the rate constant of the O→bR transition does not show the analogous pH dependence [13,175].

4.4. pH dependence of pathways of photochemical conversions of bacteriorhodopsin: origin of branching and parallel photocycles

The protonation states of Asp85 and the proton release complex (designated as X in Fig. 4) determine the pathway for the photochemical conversion of bR. The kinetics and the accumulation of specific intermediates are greatly affected by the pK_a of the PRC and Asp96 in the photocycle (Table 1). At very low pH (<2) where the proton acceptor Asp85 is protonated, and pigment is converted to the blue membrane absorbing at 603 nm, no M intermediate is formed and no proton transfer takes place [90,93,177]. At pH between 2 and 5.8 proton release is delayed until the O→bR transition [65,82]. The transition between fast and slow proton release occurs with pK_a 5.8 (in 100 mM salt) which is the pK_a of the proton release group in M [65]. In the pH range between 5.8 and 9 a decrease in the rate constant of the N→O transition and proton uptake takes place which results in a decrease in the fraction of the O intermediate and an increase in the fraction of N (Table 1). At higher pH the formation of a slightly red-shifted alkaline form of bR, bR_a, occurs with the ca. pK_a 9–9.5 [61]. This transition is most likely caused by deprotonation of the PRC in the initial (unphotolyzed) state. The alkaline form, bR_a, undergoes a photocycle which is characterized by fast M formation but no fast proton release [72]. The O intermediate is hardly detectable at high pH because of the slowing of reprotonation of Asp96 in the N→O transition [13,16,151]. In addition to that, a branch bypassing O intermediate has been proposed [35]. This branch might be caused by the deprotonation of Asp85 already in N [13]. At very high pH of (about 11) a nonpumping branch from M to bR due to deprotonation of Asp96 in the ground state becomes likely [178]. The scheme in Fig. 4 emphasizes just the main pH-dependent routes. It does not show substates of the K, L, N and O intermediates that might be important for the phototransduction mechanism. It does not include reactions that occur upon photoexcitation of intermediates [109].

5. Concluding remarks

Recent years have brought many exciting developments in the structural studies of molecular basis of light-induced proton transport by bacteriorhodopsin. They confirmed several predictions on the mechanism of the proton pumping reactions of bR and provided a new structural basis for the future studies. Despite the progress in understanding the main steps of proton transport in bR, many key issues at the heart of the mechanism of this pump await their solution on the molecular level. To name just a few: the mechanism of proton transport from the Schiff base to Asp85, which is a step common also to archaebacterial sensory rhodopsins and vertebrate rhodopsin; the exact source of the proton which is released into the bulk upon deprotonation of the Schiff base; the mechanism of the transient decrease of the proton affinity of Asp96 to reprotonate the Schiff base; the mechanism of transfer of this proton to the Schiff base, the mechanism of subsequent reprotonation of Asp96; and mechanism of thermal reisomerization during the photocycle.

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