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Review

Electrogenic processes and protein conformational changes accompanying the bacteriorhodopsin photocycle

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Abstract

The possible mechanisms of electrogenic processes accompanying proton transport in bacteriorhodopsin are discussed on the basis of recent structural data of the protein. Apparent inconsistencies between experimental data and their interpretation are considered. Special emphasis is placed on the protein conformational changes accompanying the reprotonation of chromophore and proton uptake stage in the bacteriorhodopsin photocycle.

Keywords: Bacteriorhodopsin; Proton transport; Photocycle; Conformational change

1. Introduction

Bacteriorhodopsin (bR) is a light-driven proton pump (for reviews, see [1–3]). After absorption of a photon, it undergoes a cycle of chemical conversions, which are accompanied by the transmembrane transfer of one proton, and includes the following main steps:

$$bR \overset{h\nu}{\leftrightarrow} K \leftrightarrow L \leftrightarrow M_1 \leftrightarrow M_2 \leftrightarrow N \leftrightarrow O \rightarrow bR \quad (1)$$

Light-induced all-*trans*→13-*cis*-isomerization of the retinal chromophore during the bR→K phototransition initiates changes in the environment of protonated Schiff base and Asp85, the primary proton acceptor which is the part of the complex counterion including also Arg82 and Asp212 residues. This change in the environment results in the transfer of a proton from the Schiff base to Asp85 during the L→M transition. At neutral pH, this transition is accompanied by proton release to the extracellular surface of the purple membrane from the proton release group X. At low pH, proton release to the medium occurs during the last photocycle stage (the O→bR transition).

The Schiff base is reprotonated during the M→N transition from the intraprotein donor Asp96, which is located between the chromophore and cytoplasmic surface of the membrane. The N→O transition is accompanied by proton uptake from the cytoplasmic surface, reprotonation of Asp96, and retinal reisomerization. Deprotonation of Asp85 occurs during the O→bR transition. The proton from Asp85 is transferred to the proton release group at neutral pH, or to the extracellular surface at low pH possibly through the same proton release group [4], which explains the delay of the O decay at low pH. Mod-

Abbreviations: bR, bacteriorhodopsin

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ification of the proton release group through mutation of any of its key components (Arg82, Glu204, Glu194) causes a significant decrease in the rate of the proton release from Asp85 [5–8].

Flash-induced photochemical conversions of bR and proton transport are accompanied by charge redistribution which can be measured using either the electrically oriented purple membrane immobilized in polyacrylamide gel or purple membrane adsorbed to a planar lipid membrane (for review, see [9]). Both systems give similar (at least qualitatively) results on the relative amplitudes and kinetic parameters of transmembrane charge separation [10–18]. Recently, the first attempt of detecting the intramolecular charge displacement in three dimensions was undertaken [19]. The flash-induced voltage response from transmembrane charge separation comprises the following three main phases: (i) a 'negative' one, whose amplitude does not exceed 5% of that of the whole response and with a direction opposite to the response to the continuous light, (ii) a 'positive' microsecond phase, associated with M intermediate formation, whose amplitude is 10–30% of the whole electric response according to different estimates, (iii) a 'positive' millisecond phase, associated with regeneration of the bR ground state. Certainly, the interpretation of the electrogenic phases is complicated by the possibility that in addition to the proton, some charged amino acid side chains can also move during the photocycle, and by the absence of information about local and transiently changing dielectric constants of the protein.

2. Electrogenic events associated with early stages of photocycle

The 'negative' phase contains two components with comparable amplitudes. One of them is very fast. The rate of the fast component is higher than the time resolution of most measuring systems. Only for oriented purple membrane films can its formation rate be measured with high time resolution [20]. It has been shown recently that the time constant of this component is 2.5–5 ps [21]. It is obvious that this component is due to the formation of the K intermediate. The second 'negative' component with a time constant of 1–2 μs is associated with L intermediate formation. Only these two 'negative' phases are preserved in the blue forms of the D85N, D85S, D85T and D212N mutants incapable of proton transport [22–24], as well as in the blue acid form of the wild-type bR [25–27] with protonated primary proton acceptor Asp85.

It is interesting to discuss the possible origin of these negative phases using the recently determined high-resolution three-dimensional structures of the bR ground state [28,29] and its low-temperature K [30] and M (M_N) [31] states. Although the structure of low-temperature K may differ from the structure of the K intermediate at room temperature, the generation of the 'negative' phase is observed at both room and low temperatures [32]. Apart from changing the NH bond direction (initially facing towards the extracellular surface), photoisomerization of all-*trans*,15-*anti*-retinal to 13-*cis*,15-*anti*-configuration in K intermediate results in a certain (<1 Å) shift of the N position toward the cytoplasmic surface. In the M intermediate, the change in the retinal position seems to be more pronounced. The polyene chain between C-10 and the Schiff base nitrogen is moved toward the cytoplasmic side. The C-14 atom undergoes the largest relative displacement (1.7 Å), whereas the movement of the β-ionone ring is relatively minor. This changes the angle between the conjugated π-system of the retinal and bilayer plane from 20.2° in bR to 29.6° in M. This retinal displacement seems to be the main cause of generation of the 'negative' phases associated with K intermediate formation as well as with L intermediate formation accompanied by torsion relaxation of retinal. However, displacements of some other residues should also contribute to this electrogenesis. Indeed, it would be otherwise difficult to explain the appearance of the fast 'positive' phase induced by photoexcitation of the D85N mutant form with the deprotonated Schiff base [33].

It is interesting to note that the amplitude of the 'negative' phase of the wild-type bR increases 2–3-fold as a result of formation of the blue acid form of bR at low pH. It was suggested that negatively charged Asp85 in bR at neutral pH partially compensates the charge redistribution induced by retinal isomerization [34]. Indeed, it was found [30] that the Schiff base reorientation in the K intermediate dislocates a key water molecule, which in the ground

state is sandwiched between the positively charged Schiff base and the negatively charged carboxylate of Asp85. The breaking of hydrogen bonding interaction between this water and Asp85 enables Asp85 to move closer to the position originally occupied by the Schiff base. Note that the 'negative' phase is observed also in the photoresponse of halorhodopsin, but only in the absence of Cl^- [34]. Probably, the greater mobility of chloride ion in comparison with Asp85 in bR provides full electrical compensation of this process in the chloride-binding form of halorhodopsin. Formation of the acid purple form of bR, which is due to binding of Cl^- (or some other anions) at low pH, is accompanied by further increase in the amplitude of the 'negative' phase. The amplitude of the phase in this case can reach as much as 40% of the overall amplitude of the electric response of the purple form at neutral pH [24–27]. The kinetic parameters of this 'negative' phase depend on pH and anion species [25–27]. It is suggested that the increase in the amplitude is due to appearance of the electrogenic process of some intraprotein proton movement [27] or of anion movement [24] from the Schiff base in the extracellular direction as a result of retinal isomerization-induced destabilization of an anion serving as a counterion for the protonated Schiff base. In addition to this process, a small 'positive' component is detected in the photoresponses of the acid purple form and the D85S and D85T mutants [24] at high Cl^- concentration. Probably, this phase is the low-efficiency transmembrane translocation of Cl^- in the cytoplasmic direction in accordance with the earlier photocurrent data for the acid purple form [35] and steady-state measurements in these mutants [36,37].

3. Electrogenic events associated with the M intermediate formation

Using two different approaches to the titration of Asp85, (a) direct absorption titration (determination of blue acid form concentration) and (b) measurement of pH dependence of thermal isomerization of the chromophore (dark adaptation) which is catalyzed by the protonated form of Asp85, it was found that the shape of the titration curves is rather complicated. These curves can be interpreted as coupling between the protonation states of Asp85 and the proton release group [6,8,38–40]. Protonation of Asp85 decreases the pK of the proton release group, whereas deprotonation of the proton release group increases the Asp85 affinity for protons. Deprotonation of the proton release group in the bR ground state at high pH (p$K \approx 9.5$) results in acceleration of the M intermediate formation, which is possibly due to the increase in the proton affinity of Asp85. During photocycle, the pK value of the proton release group decreases to approx. 6. Thus, at neutral pH proton is released virtually simultaneously with the $L \rightarrow M$ transition, whereas at low pH, the proton release is delayed until the $O \rightarrow bR$ transition [41]. Coupling of Asp85 and the proton release group permits to describe the events associated with dark adaptation, proton release during photocycle, and titration of bR. However, the structure of the proton release pathway includes more than two groups. According to recent description of the light-induced Schiff base deprotonation and the proton affinity of Asp85 in the R82H mutant, the third protonatable group should be included in this pathway [42].

According to the bR structure resolved at atomic (1.55 Å) resolution, an extensive three-dimensional hydrogen-bonded network of protein residues and seven water molecules leads from the buried retinal Schiff base to the membrane surface [28]. Deprotonated Asp85 and Asp212 are located near the Schiff base, Glu204 and Glu194 are located closer to the extracellular surface, whereas positively charged Arg82 has the intermediate location. Several other residues (Thr89, Tyr57, Tyr185, Tyr79 and Ser193) provide stabilization of this network. The network seems to stabilize the separated charges at the active site in the unphotolyzed state, and to provide the structural basis for the coupling between the protonation states of Asp85 and the proton release group. However, the origin of the proton released to the medium is still uncertain. Now, at least three suggestions are discussed in the literature. It was suggested that proton might originate from a water molecule hydrogen bonded to Arg82 [43]. Another possibility is the proton release from a hydrogen-bonded continuum of water molecules in which the proton is delocalized [44]. The third suggestion is that the released proton is shared by Glu194 and Glu204 in the unphotolyzed state [45]. In any case, the hydrogen-

bonded network is shielded from the extracellular interface by Ile78 and Leu201. Therefore, the proton release to the medium is thought to be an electrogenic process.

Indeed, Misra [46] observed a 2-fold decrease in the ratio of microsecond photocurrent area in bR oriented in polyacrylamide gel to the M amplitude at low pH with pK 6.1. It was suggested that although the microsecond electrogenesis at low pH is due to the proton transfer from the Schiff base to Asp85, an additional electrogenesis (proton transfer from Glu204 to the water phase) takes place at neutral pH.

However, the data discussed above are inconsistent not only with the results obtained in a different system (purple membrane attached to a planar lipid membrane) [25,47] but also with analogous experiments with oriented purple membranes [14,18,48]. According to these findings, the amplitude of the microsecond electrical component does not change significantly within the pH range from 5 to 7. We observed an insignificant (10–15%) decrease in the amplitude of the microsecond phase induced by the pH decrease from 7 to 5 [49]. This roughly corresponds to the decrease in the amplitude of the M intermediate. Although the decrease in the M amplitude at low pH with pK corresponding to pK of the proton release group is especially pronounced in the D115N/D96N and D96N mutants [41], it is also observed in the wild-type bR [50]. This pH dependence of the M amplitude is explained in the framework of the following photocycle scheme:

$$\leftrightarrow L \leftrightarrow M_1 \leftrightarrow M_2 \leftrightarrow N \leftrightarrow \quad (2)$$

where the $M_1 \leftrightarrow M_2$ equilibrium depends on the protonation state of the proton release group shifting virtually irreversibly toward M_2 at neutral and alkaline pH [41,51,52]. According to this scheme, pH decrease from 7 to 5 induces not only a decrease in the amplitude of the microsecond phase but also an increase in the ratio of amplitudes of the millisecond and microsecond phases. Indeed, it was shown in our experiments with the wild-type bR that this ratio at pH 5 is approx. 20% higher than at pH 7 [53].

Note that Rammelsberg et al. [44] did not find any amplitude or kinetic difference between the flash induced infrared (1800–1850 cm^{-1}) continuum absorbance changes at pH 5 and pH 7. It was suggested that these absorbance changes are due to the intramolecular proton transfer via an hydrogen-bonded network to the surface of the protein. Inasmuch as the majority of the electrical data also does not indicate significant differences between pH 5 and 7, it is probable that proton is released from the surface of the protein, and it is an electrically silent process.

We observed a 2-fold decrease in the magnitude of the microsecond photovoltage generation coupled to M intermediate in E204Q in comparison to the wild-type protein [49]. The same relation was found in [46]. It was concluded that deprotonation of Glu204 is an electrogenic process, and its electrogenicity is comparable to that of the proton transfer from the Schiff base to Asp85. However, we observed only one distinct component in the pH dependence of the microsecond electrogenic phase corresponding to protonation of Asp85 in the bR ground state and transition of the neutral pH purple form to acid blue form. Increase in the pH value results in an approx. 2-fold decrease in the amplitude of the microsecond phase with pK equal to the pK value of the proton release group in unphotolyzed state [47,49]. An unusual and yet unexplained feature of the E204Q mutant is the disappearance of the microsecond electrogenic phase at high pH [49].

It was suggested that Glu204 functions as the intermediate proton donor rather than the terminal proton release group, and that the pK of Glu204 in the M state is close to the pK of Asp85 in the bR ground state and far below the pK of the terminal proton release group X (approx. 6). Moreover, this group X should be deprotonated in the bR ground state, and its pK should increase during the M intermediate formation and deprotonation of Glu204. This interpretation is in agreement with the conclusion drawn by Dioumaev et al. [54] from the experiments with the E194D mutant. It was shown that the pK value of Asp194 in the bR ground state is about 3. FTIR data indicate transient protonation of Asp194: its protonation correlates with the proton transfer from the Schiff base to Asp85, whereas deprotonation coincides with the appearance of a proton at the surface, as detected with the covalently bound fluorescein. The signal amplitude decreases with increasing pH with the pK value of about 9, which is likely to be the ground state pK of Glu204, the proton donor to Asp194.

However, it is possible that conclusions made on the mutants are not fully applicable to the wild-type protein. FTIR data did not reveal any change of the protonation state of Glu204 and Glu194 during the wild-type bR photocycle [44,55]. Rammelsberg et al. [44] showed that in contrast to the wild-type protein and E204D mutant, the E204Q mutant does not demonstrate any infrared continuum absorbance changes. The authors concluded that Glu204 is a factor of stabilization of the intramolecular hydrogen-bonded chain, rather than a component of the proton release group, which directly participates in the proton transfer. Moreover, in the bR ground state, both Glu204 and Glu194 are equidistant from the membrane surface [28], which contradicts the possibility of the electrogenic character of the proton transfer between Glu204 and Glu194. However, it cannot be excluded that the X group mentioned above [49] is not Glu194 but some other component which has not been identified yet.

Another explanation of the nature of the microsecond electrogenic phase was put forward by Dickopf and Heyn [47]. According to this suggestion, at neutral pH the movement of the positively charged guanidinium group of Arg82 from a position close to Asp85 to a position near Glu204 gives a substantial contribution to the microsecond electrical phase. At alkaline pH, deprotonation of Glu204 results in localization of Arg82 near the negative charge of Glu204 in unphotolyzed bR as well as throughout the photocycle [47]. Therefore, this process results in a decrease in the amplitude of the microsecond electrical phase. Recent structural data [31] confirm the possibility that movement of positively charged Arg82 may contribute to electrogenesis. Comparison of the M intermediate structure with that of the ground state indicates the movement of Arg82 by 1.6 Å toward the extracellular surface during the M intermediate formation. NMR data also indicate that Arg82 may function as an information mediator, which transmits the information of the protonation of Asp85 to the extracellular surface, where Glu204 and Glu194 are located [56]. Restoration of the purple form but not early proton release in the R82Q mutant by guanidinium-HCl was also taken as evidence of the movement of the side chain of Arg82 during the photocycle and proton release reaction [57]. Thus, the investigation of the Arg82 mutant is of special interest for the understanding the role of Arg82 in potential generation and proton release.

It is interesting that replacement of Glu204 or Glu194 by aprotic residues results in elimination of the 'early' proton release that precedes proton uptake at any pH: proton release always follows the proton uptake ('late' proton release) and proceeds concurrently with the $O \rightarrow bR$ transition [7,8]. Thus, Glu204 as well as Glu194 seem to be components of the proton release group. The 'early' proton release is also absent in R82A mutant [6]. However, in R82Q the relative amounts of the two types of proton release ('early' and 'late') are pH dependent [58]. In the wild-type bR, the pK of the proton release group decreases from approx. 9.5 in the unphotolyzed pigment to approx. 5.8 in the M intermediate, leading to early proton release at neutral pH. In the R82Q mutant, the respective values of pK of the proton release group are approx. 8 and 7.5. The time constant of early proton release is increased from approx. 85 μs in the wild type to 1 ms in R82Q (at pH >8). This was attributed to the increase in the pK of the proton release group in the M state, which in turn explains the uncoupling of the proton release from the M formation. The component corresponding kinetically to the early proton release in R82Q at high pH was also observed as a photocurrent. Moreover, the areas under the microsecond curve corresponding to the $L \rightarrow M$ transition and 1 ms photocurrent phase were shown to be approximately equal at pH 9.0. In my opinion, the origin of the early proton release may differ from the same process in the wild-type bR. Although in the wild-type bR p$K \approx 5.8$ of the proton release group reflects the deprotonation of some surface group, in the mutant this process should be associated with the intraprotein charge transfer. Thus, this pK may be associated either with the intraprotein proton donor (for instance, this may be pK of Glu204 in the M state) or with the proton acceptor (for instance, this may be pK of Glu194 in the ground state). In any case, the data discussed above indicate that although Arg82 indeed participates in the transmission of information about protonation of Asp85 to the proton release group, it is not an absolutely necessary component. It is interesting to compare the electrical data with our photovoltage data on R82Q mutant [59]. The amplitude of the R82Q photovoltage signal within 100 μs after

flash is approx. 2 times smaller that the sum of microsecond components of the wild-type signal. Thus, the contribution of the Arg82 movement to the electrical signal may be rather small, but, of course, further experiments on this mutant are necessary for clarifying this point.

It was mentioned above that deprotonation of proton release group in the unphotolyzed state of all-*trans*-bR results in acceleration of the M intermediate formation, which may be explained by an increase in the p*K* of the proton acceptor Asp85. Possibly, the same mechanism underlies the appearance of the proton transport ability in the photocycle of dark-adapted form 13-*cis*-bR. At neutral pH, the photocycle of 13-*cis*-bR does not include formation of an M intermediate and is not accompanied by proton transport. Nevertheless, the photoelectric response of 13-*cis*-bR includes formation of a 'negative' phase resembling that of the all-*trans*-bR response and indicating the similarity of the photoinduced charge separation in the photocycles of both pigments [10,25,26,60–62]. In addition to the fast 'negative' component, which is probably associated with the bathointermediate formation in the 13-*cis*-bR photocycle, a second 'negative' component with the time constant 10–30 μs is found in the wild-type bR and in the R82K and R82Q mutants [61,62]. The possibility of charge redistribution in the outward proton pathway during this microsecond phase is discussed in [62]. At neutral pH, the 'positive' microsecond phase is absent in the photoelectric response of 13-*cis*-bR, but a distinct millisecond overshoot of the initial zero level is found [63,64]. Its rate constant is similar to that of the bathointermediate relaxation, and it was interpreted to be a consequence of charge redistribution during reconversion of all-*trans*-bR into 13-*cis*-bR (this redistribution corresponds to the shift of one positive charge in the extracellular direction to approx. 0.5 of the membrane thickness). Increase in pH induces appearance of the M intermediate [63–65] and proton transport [63,64] in the photocycle of 13-*cis*-bR, with a p*K* slightly (0.3–0.4 pH unit) lower than the p*K* of the proton release group in the unphotolyzed state of all-*trans*-bR [66]. Note that p*K* of Asp85 in 13-*cis*-bR is also approx. 0.5 unit less than the p*K* of Asp85 in all-*trans*-bR [67]. The photovoltage response of 13-*cis*-bR in the bR proteoliposomes under conditions of equal efficiencies of the M intermediate formation in the photocycles of 13-*cis*- and all-*trans*-bR, is characterized by an approx. 2-fold lower contribution of the microsecond phase as compared to the response of neutral all-*trans*-bR form [63,64]. Thus, the microsecond phase of 13-*cis*-bR is similar to that of the alkaline all-*trans*-bR form [49].

The kinetics of the M intermediate formation deviates from single exponent behavior, and was explained within the framework of Eq. 2 where $M_1 \rightarrow M_2$ transition is virtually irreversible at high pH and becomes reversible within the pH region near p*K* of the proton release group. Ludmann et al. [18] analyzed the photocurrent signal within the framework of this scheme and came to the conclusion that the electrogenicity of M_1 is 2–3 times smaller than that of M_2, both of them being pH independent within the pH range 4.5–9. It was suggested that conformational changes of the protein, consisting of tilting of the F and G helices, contribute to the positive electrogenesis in the $M_1 \rightarrow M_2$ transition. However, we did not find any significant difference between the contribution of the microsecond phase to the overall electrical response in the wild-type protein and D38R mutant (our unpublished data). According to X-ray [68] and electron [69] diffraction data, the M intermediate formation in the D38R mutant is not accompanied by large protein structural changes at neutral pH. Nevertheless, the M intermediate formation is accompanied by early proton release [70]. Thus, the M_2 intermediate formation is not necessarily associated with large conformational changes, and it seems probable that the additional positive electrogenesis of the $M_1 \rightarrow M_2$ transition gives rise only to charge redistribution over the proton outward pathway. The simplest explanation of the mechanism underlying Eq. 2 is increase in p*K* of Asp85 during the $M_1 \rightarrow M_2$ transition. In the late M intermediate, it is higher than 10.5 [71]. Thus, positive electrogenesis is probably associated with the process leading to an increase in p*K* of Asp85 (shift of Arg82 in the extracellular direction, or some other changes in the hydrogen-bonded network in the extracellular proton channel). However, additional experiments are required for clarifying these processes, because the conclusion on the electrogenicity seems to rely first of all on kinetic analysis. Ludmann et al. [72] reported a time constant of

< 10 μs for the first component of the M intermediate formation. Butt et al. [16] using the same photocurrent method found two components (50 and 200 μs) with equal electrogenicities. Inasmuch as the optical components with the time constants 50 and 150 μs and equal amplitudes at 419 nm were measured in the same experiments, one may conclude that the $M_1 \rightarrow M_2$ transition is electrically silent. Note that Althaus and Stockburger [50] reported that the M intermediate formation falls in two kinetic components with characteristic times of 60 and 150–180 μs, whereas kinetic components of 3, 40 and 130 μs were obtained by Rodig et al. [73]. In the D96N mutant, the pH dependence of the electrogenicity of M_2 was found [74]. However, formation of the M intermediate in D96N includes generation of the M_1, M_2 and M_N intermediates [75,76], thus fitting the optical and electrical signals by Eq. 2 seems to be oversimplified, because this scheme consists of only two states.

The electrogenic character of the $M_1 \rightarrow M_2$ transition is in agreement with the conclusion of Nagel et al. [77] that voltage dependence of proton pumping by bacteriorhodopsin is regulated by the voltage-sensitive ratio of M_1 to M_2. Earlier, slowing down of the M intermediate decay by membrane potential was usually regarded as the result of the slowing down of proton transport through the cytoplasmic channel against the potential [78–83]. Nagel et al. [77] found that at any potential, two processes with different time constants for the $M \rightarrow bR$ decay of 5 and 20 ms are observed. At the pump-inhibiting potentials, a third, long-lasting process with a time constant of about 300 ms at neutral pH is observed. The rate of this process does not depend on the electrical potential but depends on pH. The two fast processes were assigned to the decay of M in the normal pump cycle including reprotonation of the Schiff base via the cytoplasmic side, whereas the third slow process was interpreted as the M_1 decay proceeding without net pumping by reprotonation of the Schiff base from the extracellular side [77]. In my opinion, if this M_1 state is the common intermediate appearing in the photocycle rather than the special form induced by electric field, one should consider that the back reaction $M_1 \rightarrow bR$ may proceed even in the absence of membrane potential. Thus, this back reaction should be observed under inhibition of the forward reaction, and these data may be explained without suggestion of the existence of two M states. However, potential independence of the back $M \rightarrow bR$ transition can hardly be consistent with the fact that the electrogenicity of the M intermediate formation is at least 20% of the overall bR electrogenesis. It is easy to assume that membrane potential has a strong effect on the equilibrium between M_1 and M_2 which is due to the high electrogenicity of the $M_1 \rightarrow M_2$ reaction, whereas the $M_1 \rightarrow bR$ reaction is virtually independent on membrane potential, because of low electrogenicity of the $L \rightarrow M_1$ transition. In any case, it is necessary to suggest that membrane potential affects the forward $M_2 \rightarrow bR$ reaction by slowing it down. Otherwise, it would be necessary to suggest that membrane potential irreversibly inhibits the $M_1 \rightarrow M_2$ transition. Owing to the electrogenic character of the $M \rightarrow N$ transition and the reversibility of this reaction, membrane potential should induce a decrease in the amplitude of the fast component and have virtually no effect on its time constant. The slow component determined by the electrogenic $N \rightarrow bR$ should appear with time constant depending on the membrane potential. The absence of this potential-dependent component is due to the fact that the overall M decay is determined by the faster process – back $M_1 \rightarrow bR$ reaction. Note that slowing down of the electric potential generation by preceding flashes of varying repetition rates and intensity was observed in [81]. This effect was partially abolished by a protonophore.

4. Electrogenic events associated with the M intermediate decay

The main portion of membrane potential is generated during the millisecond phase. Kinetic components associated with M and N intermediate decay were revealed in this phase in the wild-type bR [17,18,63]. In the presence of azide, the millisecond phase of the D96N mutant has two components with comparable amplitudes: one component kinetically coincides with the M decay and proton uptake, and the second correlates with the N intermediate decay [84,85]. In addition to the electrogenic reactions associated with proton transport through the cytoplasmic proton channel, the millisecond phase includes

the electrogenic reactions associated with the O→bR transition, i.e. with proton transfer from Asp85 to the deprotonated part of the proton release group in the wild-type bR or to the membrane surface in the mutant E204Q. This possibly explains the greater contribution of the millisecond phase to the overall response in the latter case [49]. According to Ludmann et al. [18], the electrogenicity of the O→bR transition at low pH is similar to the electrogenicity of the L→M transition. The sum of these components exceeds 40% of the electrogenicity of the transmembrane proton transport. Taking into account that the Schiff base is located near the center of the membrane, these data indicate that the local dielectric constant of the outward proton channel differs only insignificantly from that of the inward proton channel, in spite of the fact that the outward pathway is enriched with charged and hydrophilic residues, whereas the inward pathway contains mainly hydrophobic residues [86].

The electrogenic nature of the O→bR transition is confirmed by the phenomenon of the O intermediate stabilization by membrane potential in liposomes containing bR [87]. It was suggested that membrane potential affects the equilibrium between the bacteriorhodopsin ground state (the proton release group is protonated and Asp85 is deprotonated) and the O intermediate (Asp85 is protonated and the proton release group is deprotonated) stabilizing the latter by changing the relative affinity of Asp85 and the proton release group for H^+. Note that the bathochromic shift of the absorption maximum in oriented purple membrane films is induced in the dark by an external electric field of a direction coinciding with that of the transmembrane field generated by the bR proton pump [88].

The slow (millisecond) component of the photoelectric response, including electrogenic reactions of proton transfer from Asp96 to the Schiff base and reprotonation of Asp96 by proton adsorbed on the cytoplasmic surface, constitutes more than 50% of the whole response. However, the relative contribution of these two reactions to electrogenesis was not determined. Ludmann et al. [18] observed the pH dependence of the electrogenicity of the N intermediate and suggested that the absence of the well-determined location of a proton donor for Asp96 can be regarded as one of the possible explanations. However, this unusual result can merely be explained by the photocycle model which was used as the best for describing the photocycle [72]. At high pH the authors suggest the direct transition N→bR without formation of O intermediate. According to their model, the rate of the O→bR transition is decreased at high pH and its rate at any pH is slower than the N→O transition. But another approach was used recently by Balashov et al. [4]. According to their model, the O→bR transition at high pH proceeds much faster than the N→O transition. The decrease in the rate of the later process results in slowing down the Asp96 reprotonation, thereby allowing to explain the decrease in the measured fraction of O without the suggestion of a modification of the photocycle route.

5. Protein conformational changes during photocycle

The problem of the transition between two intermediates called M_1 and M_2 has been extensively discussed in the literature in recent years. This transition is thought to be associated with the 'reprotonation switch' that changes access of the Schiff base from the extracellular side to the cytoplasmic side. The scheme $L \leftrightarrow M_1 \rightarrow M_2$ was originally proposed to explain the complicated kinetics of the M intermediate formation [41,51,52,89,90].

Probably, the mechanism of proton transport along the outward proton pathway which represents the hydrogen-bonded network differs from that along the inward proton pathway. Although the extracellular domain of the protein is enriched with charged and hydrophilic residues, the cytoplasmic domain (which contains the intraprotein proton donor Asp96) consists of mainly hydrophobic residues [86]. Skulachev [85,91] suggested that formation of the second M form is accompanied by conformational protein changes resulting in approaching of Asp96 to the Schiff base and formation of the water cleft between the cytoplasmic surface and Asp96, thereby facilitating reprotonation of the latter. Because the photocycle in the D212N mutant is accompanied by protein conformational change and by deprotonation of Asp96 against the background of protonated Schiff base [92], it was suggested that the proton transfer from Asp96 to the Schiff base in the

wild-type photocycle is driven also by such a decrease in the pK of Asp96. Recently, a decrease in the pK of Asp96 from >11 [93] in the ground state to approx. 7.1 in the N intermediate was directly shown by FTIR spectroscopy [55]. The photocycle inhibition induced by osmotically active solvents [94], low humidity [95], and high hydrostatic pressure [96] suggested that the proton transfer from Asp96 to the Schiff base, and perhaps also reprotonation of Asp96, are controlled by changing dipole environment. The change in the dipole environment is thought to be provided by changes in the extent of hydration of the cytoplasmic domain as a result of 'opening' of the interhelical cavity to the aqueous medium [97].

The FTIR difference spectra in the amide I (around 1650 cm^{-1}) and amide II (around 1550 cm^{-1}) regions, which are sensitive to conformational changes of proteins, revealed a definite difference between the M_1 and M_2 states (see discussion in [3,98]). Further changes in the FTIR spectra are observed during the N intermediate formation. FTIR spectroscopy allowed to observe the intermediate M_N, which can be accumulated at high pH during the photocycle of the D96N mutant [99] or wild-type bR in the presence of guanidine chloride [98]. The protein structure in the intermediate M_N is similar to that in the N intermediate, except for deprotonated Schiff base [98,99].

Given the two-dimensional crystalline nature of purple membrane, the most direct evidence of global conformational changes was obtained using electron [69,100–104], neutron [105,106] and X-ray [98,107–109] diffraction. However, a comparative interpretation of these data is hampered by different conditions for trapping the intermediates and the absence of their identification by other methods. The protein structure in some mutants is modified even in the unphotolyzed state, and this brings into question the adequacy of the data obtained in these mutants. Nevertheless, some general conclusions can be drawn. First of all, the L intermediate formation is not accompanied by conformational changes [104]. Formation of M_1 intermediate seems to proceed also without significant conformational changes. Stabilization of the M_1 state and interruption of the $M_1 \rightarrow M_2$ transition were observed at low temperature [98,100,104], low humidity [98,106], or after replacement of Asp38 by Arg [68,69]. Further formation of M_2 and M_N is accompanied by global protein conformational changes. Although there is general agreement that the main light-induced changes are mainly confined to the area of B, F and G helices, the estimates of relative intensity of these changes provided by different authors differ from each other. The structural changes take place on the cytoplasmic side of the membrane. Note that according to the crystal structure of the wild-type bR [28], the cytoplasmic part of the protein is considerably less buried in lipid, which may be relevant to the fact that the cytoplasmic rather than the extracellular part undergoes large conformational changes during the photocycle. The literature on the problem of similarity between M_2 and M_N states is slightly controversial. In the N intermediate trapped in the F219L [102] and F171C [108] mutants, the main changes are observed near the F and G helices. It is suggested [102,108,109] that changes near the B and G helices take place during formation of the early M (M_2?) state, whereas movement of the F helix is observed during N or M_N formation. On the other hand, Sass et al. [98] came to the conclusion that in spite of the difference in the FTIR spectra, large-scale conformational changes near the B, F and G helices in M_2 and M_N do not differ from one another. Using the time-resolved electron diffraction [69] it was concluded that in the wild-type bR significant conformational changes (with the strongest peak near the helix G and with progressively smaller features near helices B, F and C) occur as early as 1 ms after flash at pH 6. Only a small increase in the extent of this change is observed at higher pH, and only at longer time intervals when more N intermediate is accumulated. Structural changes in the D96N mutant are virtually identical to those in the wild-type bR. Comparison of crystal structures of the unphotolyzed state and late M (M_N) [28,31] did not reveal any displacement of helices A to E. Cytoplasmic ends of helices F and G are disordered in the M state (between residues 162 and 175 in F, and between residues 222 and 231 in G).

It was shown in our experiments [75,110] that glutaraldehyde, lutetium or aluminum ions, sucrose, and glycerol, which is known to inhibit the M decay in the wild-type bR, inhibit also the azide-facilitated M decay in the D96N mutant. It was concluded that an equilibrium exists between two M forms (M_{closed} and

M_{open}), which differ from one another by the accessibility of the Schiff base to azide and, probably, also to water molecules. Note that inhibitors do not affect the pH dependence of the M amplitude with $pK \approx 5.4$, which seems to be due to the pH dependence of the $M_1 \leftrightarrow M_2$ reaction. Thus, the effect of inhibitors on the $M_1 \rightarrow M_2$ transition is not very strong. The inhibitors induce a shift of the equilibrium toward the less accessible M form. However, different inhibitors may have different mechanisms of action. For example, glutaraldehyde and lutetium ions possibly affect the conformational flexibility of the protein [75], whereas the effects of glycerol and sucrose seem to be due to a decrease in the water activity [94].

In this context, the effect of azide on the photocycle of the D96N mutant should be discussed in more detail. It is well known that hydrogen azide and some other weak acids accelerate the M intermediate decay in the D96N mutant [17,85,94,111, 112]. Therefore, they can replace missing carboxyl group of Asp96 as an internal proton donor. The pH dependences of the weak acid effects are consistent with the suggestion that their protonated forms are active species [85]. Azide is also able to accelerate deprotonation of Asp85 and Schiff base from the extracellular domain [33,112,113]. The simplest suggestion is that azide and other acids act as penetrating proton carriers (like uncouplers of oxidative phosphorylation). However, another model was discussed in [114]. According to this model, the binding of azide near Asp85 affects the proton movement by reorganizing a long-range hydrogen-bonded chain of water molecules connecting the Schiff base with the cytoplasmic surface. This chain is disrupted in the D96N mutant. As shown by Brown and Lanyi [115], this model can also explain the pH dependence of azide action, provided that the N_3^- binding efficiency is a linear function of its concentration, the bound N_3^- and HN_3 are in rapid equilibrium with the aqueous phase, and proton conduction catalyzed by the azide bound is directly proportional to proton concentration. According to FTIR data [114], even at 100 mM HN_3 one molecule of azide is protonated concurrently or faster than the M intermediate formation and deprotonated concurrently with the N intermediate decay. Thus, it seems that saturation is achieved at 100 mM HN_3. Nevertheless, according to our data, saturation of the rate of the Schiff base reprotonation is not achieved even in the presence of 1 M HN_3 at the same pH 5 [116]. Recently [117], by means of site-specific mutagenesis, nitroxide spin labels were introduced at different positions at the protein surface and in the protein interior. This allowed to determine the polarity changes along the proton pathway. The maximum of the hydrophobic barrier is found to be close to the retinal chromophore. In the presence of azide, the barrier heights of both cytoplasmic and extracellular channels are decreased. These data were considered as a support of the view that azide acts as catalysts of the formation of hydrogen-bonded networks in the proton pathway. Note that the experimental value of K_a for this effect is 10 mM, which is comparable with the value found from the reactivation of the proton transport activity of the D96N mutant, $K_a = 3$–4 mM [111,117]. However, it is obvious that the latter value measured under steady-state photocurrent study is determined by the slowest process of photocycle, the $N \rightarrow bR$ transition, which does not depend on azide, and which limits the overall rate of the photocycle at high azide concentration [85].

Our data on the action of different inhibitors on the azide-stimulated M decay in the D96N [75,110] mutant are also inconsistent with this model. Inhibition of azide-stimulated M decay, which is reversed by increase in the azide concentration, can be explained as the decrease in the azide affinity for bR. However, the similarity between the actions of these inhibitors on the M decay in the wild-type bR and azide-stimulated M decay in the D96N mutant makes this explanation rather unconvincing.

It is interesting that the protonation-deprotonation cycle of bound azide is observed at pH 5 but not at pH 7 [114]. Because at pH < 6, proton is released during the last step of photocycle rather than simultaneously with the M formation [41], it is possible that the bound azide somehow takes part in functioning of the proton release complex resembling protonation changes of Asp194 in the E194D mutant [55].

The agents that inhibit the azide-stimulated M decay in the D96N mutant do not affect the rate of the M decay at high ionic strength in the absence of azide at pH > 5 [75,110,118]. These findings were rather surprising, because they imply different mech-

anisms of the Schiff base reprotonation by a proton from water and during the azide-stimulated M decay. However, the latter process is known to be accompanied by the proton uptake from the cytoplasmic surface [85,111]. It was found that glutaraldehyde does inhibit the M decay only at low pH. The complicated pattern of the pH dependence of M decay seems to be due to the coexistence of two pathways of the M conversion. The M decay pathway dominant at pH <5 is coupled to the proton uptake from the cytoplasmic surface and proceeds through the N intermediate. The M decay pathway insensitive to inhibitors is predominant at pH >5 and is approximated by a curve describing titration of some group with a pK value close to the pK of the proton release group. It is suggested that this pathway of M decay is coupled with back proton uptake from the extracellular surface and via the M_1 and, perhaps, L intermediates. At low ionic strength, an intricate pH dependence of the surface potential and, therefore, of the surface proton concentration may impose additional complexity on the pH dependence of the M decay curve [119]. In this context, the reverse process can be compared with the photocycle of the wild-type bR at low humidity [120]. From the analysis of the photoelectric signal it was concluded that protons move forward during the L decay and return to their original place during the M decay. According to recent data, this M intermediate should be the M_1 state [98]. Unfortunately, the photoelectric responses of the D96N mutant cannot be studied at high salt concentration because of fast passive discharge. Nevertheless, the photoelectric data obtained at low ionic strength [53,121] revealed a decrease in the millisecond phase rate constant and amplitude, which is in agreement with the suggestion that increase in pH is accompanied by appearance of the back proton uptake process. These data indicate that the real difference between the rates of proton uptake in the D96N mutant and in the wild-type bR is even higher than the difference between the rate of the M decay in the D96N mutant and that of the N decay in the wild-type bR at the same pH value. The difference between proton uptake rates seems to be due to the important role of the negatively charged Asp96 residue in facilitating of proton uptake through the cytoplasmic half-channel in the wild-type protein [94].

It is well known that in the mutants with Asp96 replaced by Asn, the M intermediate formation is accompanied by a blue shift of the absorption maximum from 412 nm to 404 nm [89,116,122]. All the above mentioned inhibitors of the M decay inhibit this absorption shift. Thus, the absorption spectrum of M_{open} is shifted toward a shorter wavelength relative to the spectrum of M_{closed}. The inhibitors used do not prevent the multiphase pattern of the M formation in either mutant or wild-type protein. This may indicate that M_{closed} itself is a mixture of different conformational states. Note that at low temperature, when only M_1 is presumably formed, at least four substates of the M intermediate were found [123]. These substates differed from each other by the characteristics of back reaction induced by blue light. Our data indicate that M_{open} appears within submillisecond time domain, while, according to FTIR data, M_N appears also in submilliseconds [76]. Thus, M_{open} seems to be identical to M_N, and its structure is similar to the structure of the N intermediate. This brings up the question whether the blue shift of the M_N maximum is the property of the mutant [89,122] or the M_N intermediate of the wild-type bR has the same spectra? On the basis of our data, it can be suggested that the latter case is more probable.

It was found [124] that the maximum of the M intermediate difference spectrum in the wild-type purple membrane is at 405–406 nm under conditions [98] favoring accumulation of the M_N intermediate (6 M guanidinium chloride, pH 9.6), whereas immediately after pulse laser excitation, the maximum is observed at 412 nm. In spectra recorded 0.1 ms after the flash in the absence of guanidine chloride at pH 11.3, the maximum is also localized at 412 nm. In several milliseconds, the maximum is 5–6 nm shifted to the short wavelength region. Although this shift is similar to that in the D96N mutant, there are two main features of difference: (i) the rate of the shift is slower in the wild-type bacteriorhodopsin, and is similar to the rate of the M to N intermediate transition ($t_{1/2} \approx 2$ ms); (ii) the shift in the wild-type bacteriorhodopsin is observed at alkaline pH values which are higher than pK of the Schiff base (approx. 10.8 at 1 M NaCl) in the N intermediate with the deprotonated Asp96 [124].

All inhibitors of the M decay accelerate N decay in

the D96N mutant [125]. The acceleration of the N decay is accompanied by a blue shift of the N difference spectrum by approx. 10 nm. It is concluded that the N intermediate represents, in fact, an equilibrium mixture of two ('open' and 'closed') forms. These two forms, like M_{closed} and M_{open}, attain an equilibrium state within several microseconds [75,76,118]. The absorption spectrum of N_{closed} is slightly shifted to longer wavelengths in comparison with that of N_{open}, like in the M forms. 13-*cis*-all-*trans*-reisomerization is assumed to occur only in the N_{closed} form. Note that the protein conformation of O, the last intermediate in the photocycle, having the all-*trans*-chromophore, is similar to that in the bR ground state [126,127]. On the other hand, the long-living O-like intermediate with 13-*cis*-retinal in the L93A mutant differs from bR in the vicinity of helices C, B and G [103] but, according to FTIR data [127], it is similar to bR with respect to the structural changes around Asp96. Thus, this intermediate is similar to the N_{open} state.

Protonated azide, acetate, and formate inhibit the N decay in both the D96N mutant and the wild-type protein [125]. In my opinion, the inhibition of the N intermediate decay by azide is due to the ability of its neutral form to permeate into a cleft in the bR molecule inherent in N_{open}, thereby stabilizing this intermediate and shifting the equilibrium toward N_{open}. The logarithmic plot of the N decay rate dependence on azide concentration has a slope of about 1.6. Therefore, it can be suggested that the N_{open} stabilization is induced by binding of only 1–2 azide molecules to the protein cleft.

In my opinion, the photocycle of the D96N mutant can be described by the following scheme:

$$\leftrightarrow L \leftrightarrow M_1 \leftrightarrow M_2 \leftrightarrow M_2' \leftrightarrow M_N \leftrightarrow N_{open} \leftrightarrow N_{closed} \leftrightarrow$$

$$(O) \rightarrow bR \qquad (3)$$

In the D96N mutant, the proton uptake in the presence of azide proceeds simultaneously with the M decay ($M_N \rightarrow N_{open}$) rather than with the N decay [85]. Note that Eq. 3 seems to correctly describe not only the photocycle in the D96N mutant but also the photocycle in the wild-type bR in the presence of azide, which is known to accelerate the M decay but with lower efficiency [75,85,110,114,128]. This may be due to low concentration of M_{open} (M_N) in the photocycle of the wild-type bR, because a decrease in the p*K* of Asp96 should induce its immediate transformation into N intermediate. The intermediates M_1 and M_2 belong to the forms with low and high p*K* of Asp85, respectively. M_2' is the closed form of the M intermediate which undergoes large conformational changes. Possibly, these conformational changes result in formation of proton channel but its opening takes place during the next photocycle step. In spite of the fact that the loss of interaction of the positively charged Schiff base with its counterion induces conformational changes [129], these events are not necessarily associated with each other (see discussion of the D38R mutant considered above). Probably, the target of inhibitors of M decay is the $M_2' \rightarrow M_N$ transition. However, by analogy with the D38R mutation, their effect on the $M_2 \rightarrow M_2'$ transition should not be disregarded. Note that a decrease in the relative humidity first of all affects the $M_2 \rightarrow M_N$ transition and only after that, the $M_1 \rightarrow M_2$ transition (relevant experimental data are described in [130] and discussed in [125]).

A large body of diffraction data [68,69,98,100,104] indicate that the main conformational changes take place during the $M_1 \rightarrow M_2$ transition. The inhibitors used in [75,110] and hydrostatic pressure [96] slow down the rate of the M formation but do not affect the flash-induced M amplitude in the wild-type bR at neutral pH. The latter is probably due to the irreversibility of the $M_1 \rightarrow M_2$ transition. However, EPR spectroscopic studies of spin-labeled purple membranes [131–133] and flash-induced light scattering changes in purple membrane suspension [134,135] showed that some conformational changes also occur during the $M \rightarrow N$ transition. In addition, it was found that hydrostatic pressure strongly inhibits the M decay [96]. Ultraviolet resonance Raman study [136] revealed a hydrophobic to hydrophilic change in the environment of Trp182, which is located between retinal and the cytoplasmic surface. This change correlates with the $M \rightarrow N$ transition. It is interesting that deprotonation of the Schiff base of the D85N mutant in the dark results in protein conformational changes [129]. Further changes resembling the $M \rightarrow N$ transition take place during the proton transporting photocycle of this deprotonated form [137]. All these effects can be explained assuming that M_N and M_2 are of different conformations,

the volume of M_N being larger than the volume of M_2. Obviously, these conformational changes leading to penetration of a few small molecules into the protein are rather small scale and occur mainly near the surface. Subramaniam et al. [69] observed minor structural difference between intermediates trapped at early and late stages of the photocycle as well as between M intermediates in the wild-type bR and D96N mutant. Thus, conformational changes in M_2 can be regarded as formation of a cleft, and the further changes during formation of M_N and N, as the cleft opening and permeation of water molecules or H_3O^+ (in N state).

Eq. 3 does not include the specific stage of 'reprotonation switch', because the high p*K* of Asp85 in M_2 is a reliable defense mechanism against the reprotonation of the Schiff base from the extracellular part. The absence of the water cleft in the ground state allows deprotonation of the Schiff base only in the extracellular direction. Note that the small difference in the kinetics of the back blue light-induced reaction shows a small difference of the different M forms in respect of the spatial position of the Schiff base relative to Asp85 [138]. Thus, in this respect, Eq. 3 is similar to the 'local-access model' [139,140], but not to IST model [141] of the bR photocycle.

The photocycle of the wild-type bR in the absence of azide differs from the photocycle of the D96N mutant. At least one additional step associated with the protonation of the N intermediate ($N^{-1} \rightarrow N^0$) [142] should be added. The two forms seem to be the N_{open} states. Note that a negative charge on Asp96 stabilizes the 13-*cis*,15-*anti*-conformation of the chromophore group [143].

It is interesting that different effectors induce simultaneous acceleration of the M decay and deceleration of the N decay. These effectors are: the addition of a small amount of Triton X-100 [144] or alcohol [145] to the wild-type bR, substitution of T46 by V, introduction of bulky groups in the helix F [97], and a decrease in the intensity of the exciting flash (the 'cooperative' phenomenon) [146–148]. All these effects are thought to be due to stabilization of the 'open' N and M forms in comparison with their 'closed' states. Probably, interaction between T46 and D96 [28,149] is an important factor in the 'closed' form stabilization, because the D96 → N substitution (like the T46 → V substitution) leads to a significant delay of the N decay and to a significant increase in the concentration of the M_{open} form [75,85,110,150]. Introduction of a bulky group in the helix F may result in stabilization of the 'open' forms, whereas further cross-linking may induce an opposite effect of the 'closed' form stabilization and, hence, acceleration of the N decay and inhibition of the M decay [97]. On the other hand, substitution of R227 by Q results in deceleration of the M decay and acceleration of the N decay [150] which is probably due to stabilization of the 'closed' states in comparison with the 'open' forms.

If the volumes of 'open' forms are slightly larger than those of 'closed' forms, an increase in the hydrostatic pressure should shift equilibrium toward the 'closed' states. An increase in the hydrostatic pressure actually retards the M intermediate decay and accelerates the N decay in the T46V mutant. However, in the wild-type protein, an increase in the hydrostatic pressure retards the M decay but has no effect on the N intermediate decay at pH 10 [96]. The latter phenomenon can also be explained in the framework of Eq. 3 including the transition $N_{open}^{(-1)} \rightarrow N_{open}^{(0)}$. The effect of the shift of the $N_{open} \leftrightarrow N_{closed}$ equilibrium on the overall rate of the N decay should depend on the ratio between the rate constants of protonation of N ($N_{open}^{(-1)} \rightarrow N_{open}^{(0)}$) and reisomerization ($N_{closed} \rightarrow bR$). Slowing down of the N protonation decreases the effect of the equilibrium shift on the N decay. A significant delay of the N protonation caused by high pH or by some other factors should eliminate this effect. Therefore, the effect of hydrostatic pressure on the N decay should not be observed.

The proposed Eq. 3 offers an appropriate explanation of the 'cooperative' phenomena. Increase in the flash intensity would lead to the excitation of neighboring bR molecules, whose interaction in rigid purple membranes should shift the equilibrium toward the 'closed' states. This would result in deceleration of the M decay and acceleration of the N decay [146–148].

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