Two forms of N intermediate (N_{open} and N_{closed}) in the bacteriorhodopsin photocycle

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Abstract Glutaraldehyde, aluminum ions and glycerol (that inhibit the M intermediate decay in the wild-type bacteriorhodopsin and azide-induced M decay in the D96N mutant by stabilization of the M_{closed}) accelerate the N decay in the D96N mutant. The aluminum ions, the most potent activator of the N decay, induce a blue shift of the N difference spectrum by ~10 nm. Protonated azide as well as acetate and formate inhibit the N decay in both the D96N mutant and the wild-type protein. It is concluded that the N intermediate represents, in fact, an equilibrium mixture of the two ('open' and 'closed') forms. These two forms, like M_{closed} and M_{open}, come to an equilibrium in the microseconds range. The absorption spectrum of the N_{open} is slightly shifted to red in comparison to that of the N_{closed}. Again, this resembles the M forms. 13-*cis*-all-*trans* re-isomerization is assumed to occur in the N_{closed} form only. Binding of 1–2 molecules of protonated azide stabilizes the N_{open} form. Existence of the 'open' and 'closed' forms of the M and N intermediates provides the appropriate explanation of the cooperative phenomenon as well as some other effects on the bacteriorhodopsin photocycle. Summarizing the available data, we suggest that M_{open} is identical to the M_N form, whereas M_1 and M_2 are different substates of M_{closed}.

Key words: Bacteriorhodopsin; Photocycle; Conformational change; Proton pumping; Azide; Purple membrane; D96N mutant; *Halobacterium salinarium*

1. Introduction

Bacteriorhodopsin (bR) from *Halobacterium salinarium* is a proton pump that converts light energy into a proton electrochemical gradient across the bacterial membrane (for reviews, see [1–4]). The chromophore group of bR is a retinal covalently bound to the ε-amino group of the Lys-216 via protonated Schiff base. Light absorption induces an all-*trans* to 13-*cis* isomerization of the chromophore and a complex photochemical cycle including J, K, L, M, N and O intermediates. During L→M transition, the chromophore isomerization initiates the proton transfer in the direction to the outer membrane surface, occurring from the Schiff base to the proton acceptor group of Asp-85. The following M→N transition is coupled with the Schiff base reprotonation by the proton donor group of Asp-96 localized between the chromofore and the cytoplasmic surface. The M decay is greatly retarded in the D96N mutant. The N decay and bR initial state restoration is accompanied by reprotonation of the D96 and the chromophore 13-*cis*-all-*trans* re-isomerization.

Numerous data indicate to the heterogeneous nature of both the M [5–29] and N [30–34] intermediates. Special attention was paid to the problem of changing the accessibility of the Schiff base between the outward H^+ half-channel where Asp-85 is located and the inward H^+ cytoplasmic half-channel with Asp-96 involved.

In this group, a fast (in the submilliseconds range) equilibrium between two ('open' and 'closed') forms of the M intermediate was postulated on the basis of an inhibitory analysis of the M decay in the wild-type bR and azide-dependent M decay in the D96N mutant, [17,18,22]. The equilibrium is assumed to be shifted toward the M_{open} (M_N) with an absorption maximum <404 nm in the D96N mutant. In the wild-type bR, the concentration of the M_{open} under equilibrium is small and determines the rate of the Schiff base reprotonation and the N intermediate formation. The M_N formation in the D96N mutant photocycle proceeding in the submilliseconds time domain was recently found by FTIR spectroscopy, whereas this process is not observed in the wild-type bR [35].

In the present work, on the basis of the inhibitory analysis, it is concluded that the N intermediate, like the M intermediate, is an equilibrium mixture of the two N forms, namely N_{open} and N_{closed}, and 13-*cis*-all-*trans* re-isomerization takes place in the N_{closed} form only.

2. Materials and methods

Freshly prepared purple membrane sheets from the halobacterial wild-type ET1001 and D96N mutant strain were used. The strain was kindly provided by Prof. D. Oesterhelt (Max-Planck Institut für Biochimie, Germany).

The bR photocycle transient absorbance changes were measured using a laboratory-built single-beam spectrophotometer as previously described [10,11,17]. The time-resolved difference spectra were obtained by computer processing of 36 curves of absorption change signals measured in the 360–710 nm region with a step of 10 nm. Light flashes were provided by a frequency-doubled Quantel Nd-YAG-481 laser (wavelength, 532 nm; pulse half-width, 15 ns; energy, 10 mJ). The time-resolved absorbance change curves were decomposed into components with aid of the DISCRETE program [36].

Measurements were performed on the light-adapted purple membrane suspension at 20°C. The pH-dependence of azide acceleration of the M decay in the D96N mutant has an apparent p$K\sim5.6$ [10,18] and all experiments were carried out at pH 5.0.

Glutaraldehyde treatment of bR was performed as described previously [17,18].

3. Results and discussion

The flash-induced amplitude of the M intermediate is negligible in the presence of 1 M NaN_3 (Fig. 1A and C, curve 1) because the rate of the M decay is faster than that of its

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Abbreviations: bR, bacteriorhodopsin

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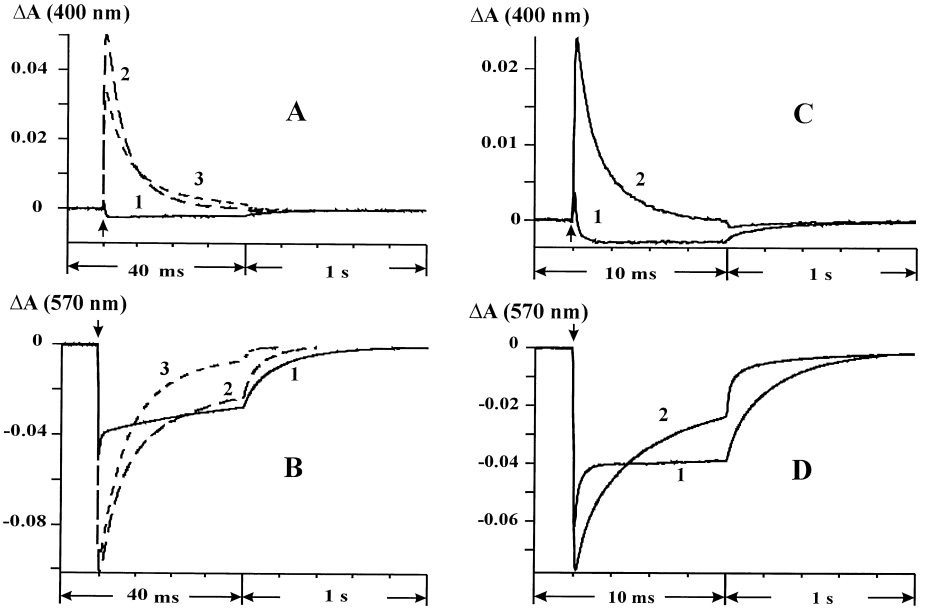

Fig. 1. The laser flash-induced optical responses in the D96N mutant. The assay medium: 1 M NaN_3, pH 5. A, B: 1, no addition; 2, 70% glycerol; 3, 2 mM $AlCl_3$. C, D: 1, untreated bR; 2, glutaraldehyde pre-treated bR.

formation at the azide concentration used [11]. The rate of the N intermediate decay in the D96N mutant is significantly slower than that in the wild-type bR [10,32] and this process can be easily measured at 570 nm due to the fast rate of the M decay (Fig. 1B and D, curve 1).

Glycerol and aluminum ions increase the M amplitude by inhibition of the M decay (Fig. 1A, curves 2 and 3). Glutaraldehyde pretreatment induces the same effect (Fig. 1C, curve 2). Since experiments were performed at a low pH, aluminum ions were used instead of the lutetium ions [17,18] since their inhibitory effects depend on pH. Whereas lutetium ions are potent inhibitors of the M decay at $pH > 6$, aluminum inhibits the M decay at a low pH [37].

All the above-mentioned inhibitors of the M decay induce the acceleration of the regeneration of the bR ground state and the N decay (Fig. 1B, curves 2 and 3 and Fig. 1D, curve 2). These effects can be explained in the framework of the model suggesting that the N and M intermediates consist of two forms, namely 'open' and 'closed' (Fig. 2). The $N_{open} \leftrightarrow N_{closed}$ equilibrium is probably fast and takes place within the submilliseconds time domain, being similar to the $M_{closed} \leftrightarrow M_{open}$ equilibrium [17,18,22]. The inhibitors shift this equilibrium towards the 'closed' states by their stabilization and thus simultaneously decrease the rate of the M decay and increase the rate of the N decay. Obviously, the latter process can be observed when the M decay is not the rate-limiting stage of the bR photocycle. This requirement is fulfilled in the D96N mutant at a high azide concentration due to the second order character of the $M \rightarrow N$ transition. The mechanism of the N_{closed} and M_{closed} stabilization seems to be the same: fixation of the protein conformation by the glutaraldehyde and aluminum ions or the water activity decrease by glycerol [17,18,22].

The following experiments confirm the proposed explanation. Fig. 3 shows a comparison between difference spectra of the N intermediate measured in the absence (1) and presence (2) of aluminum ions, the most potent activator of the N decay. Aluminum ions induce an $\sim$10 nm short wavelength shift of the N difference spectra. It should be noted that the aluminum ions do not affect the absorption maximum of the

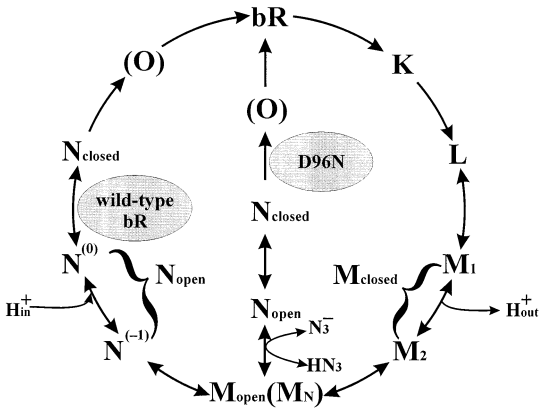

Fig. 2. Tentative scheme of the photocycles in the wild-type bR and in the D96N mutant.

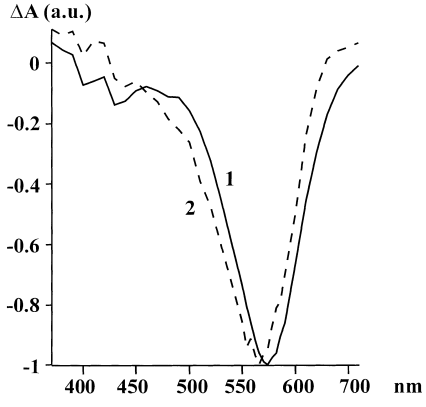

Fig. 3. The effect of $AlCl_3$ on the N intermediate difference spectrum in the D96N mutant. The difference spectra were measured in 150 ms after flash. The assay medium: 1 M NaN_3, pH 5. 1, no addition; 2, 2 mM $AlCl_3$.

bR ground state (data not shown). The absorption maximum of the N intermediate is localized near the maximum of the bR ground state but has a lower extinction coefficient [38], the data suggest that aluminum ions induce the long wavelength shift of the N intermediate absorption maximum. According to the model suggested, aluminum ions shift the equilibrium between N_{open} and N_{closed} towards N_{closed}. Thus, the N_{closed} absorption maximum should be shifted slightly towards the red similar to the shift of the M_{closed} spectrum in comparison with the M_{open} form [22].

Since azide can penetrate into the M_{open} form and serves as a proton donor for the Schiff base, its possible effect on the N intermediate was investigated. It is found that high concentrations of azide significantly inhibit the N decay at pH 5 but not at pH 7 (Fig. 4A). pH-dependence of the azide effect on the N decay indicates that the inhibitory effect is due to the protonated form (Fig. 4B). Fig. 4A and B present the time constants of a slow component of the N decay. Its contribution to the overall N decay exceeds ca. 85% at a low flash intensity and is equal to 55–60% at a high flash intensity. This component was attributed to the photocycle of the non-interacting bR molecules [31]. Note that azide induces qualitatively the same inhibition of fast components of the N decay (data not shown) corresponding to the photocycles of interacting neighboring molecules [31]. Other small weak acids (formate and acetate), that are known to accelerate the M decay, also inhibit the N decay. In spite of the fact that their ability to accelerate the M decay is much less pronounced than that of the azide, the mentioned weak acids inhibit the N decay with the same efficiency as azide (data not shown). Such relationships are probably due to some additional interprotein sterical restrictions imposed on their action as the proton donors. We suppose that inhibition of the N intermediate decay by azide is due to the ability of its neutral form to penetrate into a cleft in the bR molecule [3,10] inherent in N_{open} and thus to stabilize this intermediate as well as to shift the equilibrium towards N_{open}. The dependence of the N decay rate on the azide concentration has a slope in the logarithmic coordinate of about 1.6 (Fig. 4A). The data obtained by electron microscopy [39–42], EPR study [43] and neutron diffraction [44] indicate rather small changes in protein conformation during the photocycle. Therefore, we suppose that this slope is due to that the N_{open} stabilization is induced by binding of only 1–2 azide molecules in the protein cleft.

Noteworthy is that inhibition of the N decay by azide is also observed in the wild-type protein. It is known that azide

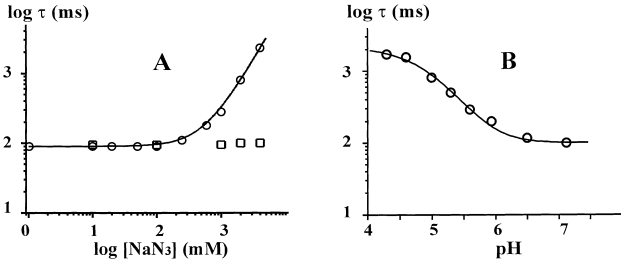

Fig. 4. The effect of NaN_3 on the N intermediate decay. A: dependence of the slow component of the N decay on the NaN_3 concentration. (○), pH 5; (□), pH 7. In all samples, the Na^+ concentration was kept at a constant level of 4 M by NaCl addition. B: pH-dependence of the slow component of the N decay rate at 2 M NaN_3.

is able to accelerate the M decay in the wild-type protein [10,17,45,46]. In addition to this acceleration (Fig. 5A), azide at a high concentration also inhibits regeneration of the bR ground state (Fig. 5B). A differential spectrum of the slow components observed in the presence of azide is typical of the N intermediate (data not shown). Thus, the conclusion on the existence of two N forms based on the experiments with the D96N mutant seems to be suitable to the wild-type protein as well. Nevertheless, the photocycle of the wild-type bR differs from the photocycle of the D96N mutant. At least one additional step associated with the protonation of the N intermediate [30] should be added (Fig. 2).

In the D96N mutant, proton uptake in the presence of azide proceeds simultaneously with the M decay but not with the N decay [10]. Note that the scheme (Fig. 2) proposed for the D96N mutant 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.

It is interesting that different effectors induce simultaneous acceleration of the M decay and slowing down of the N decay, addition of a small amount of Triton X-100 [38,47] or alcohol [48] to the wild-type bR, substitution of T46 by V or R227 by Q [49], introduction of bulky groups in the helix F [50], a decrease in intensity of the exciting flash (the 'cooperative' phenomenon) [31,32,34]. We suppose that all these effects are due to the stabilization of the 'open' N and M forms in comparison with their 'closed' states. Probably, an interaction between T46 and D96 [51,52] is an important factor in the 'closed' form stabilization since the D96→N substitution leads, just as the T46→V substitution, to a significant delay of the N decay (Fig. 1B (curve 1) and Fig. 5B (curve 1)) and

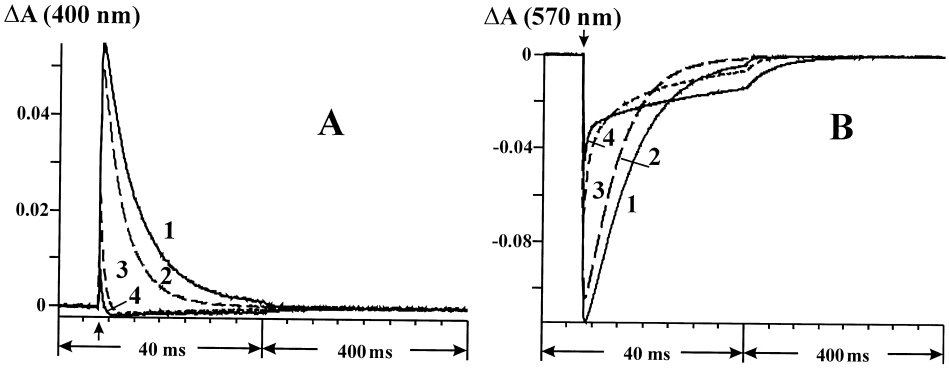

Fig. 5. The laser flash-induced optical responses in the wild-type bR. 1, 2 M NaCl; 2, 1.9 M NaCl, 0.1 M NaN_3; 3, 1 M NaCl, 1 M NaN_3; 4, 2 M NaN_3.

to a significant increase in the concentration of the M_{open} form [17,18,22]. Introduction of a bulky group in the helix F may result in stabilization of the 'open' forms, whereas following cross-linking may induce the opposite effect of the 'closed' form stabilization and, hence, acceleration of the N decay and inhibition of the M decay [50].

If the volumes of the 'open' forms are slightly larger than that of the 'closed' forms, an increase in the hydrostatic pressure should shift the equilibrium towards 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 does not affect the N intermediate decay at pH 10 [53]. The latter phenomenon can also be explained in the framework of the scheme on Fig. 2. 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 the protonation of the N ($N_{open}^{(-1)} \rightarrow N_{open}^{(0)}$ and re-isomerization ($N_{closed} \rightarrow bR$). 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 a high pH or by some other factors should eliminate this effect and, hence, an effect of the hydrostatic pressure on the N decay should not be observed.

The proposed scheme (Fig. 2) offers the appropriate explanation of the 'cooperative' phenomena. An increase in the flash intensity will lead to excitation of the neighboring bR molecules whose interaction in rigid purple membranes should shift the equilibrium towards the 'closed' states. This will result in slowing down of the M decay and acceleration of the N decay [31,32,34].

Previously [22], we came to the conclusion that M_{open} is identical to the M forms denoted as M_N or M_2. Based on the recent data, we suppose that M_{open} is identical to M_N which, according to the FTIR data, has the protein structure similar to that of the N intermediate [13], whereas M_2 as well as M_1 are substates of M_{closed}. Our data based on the spectroscopic and inhibitory analysis [17,18,22] indicate that in the photocycle of the D96N mutant, an equilibrium between the M forms is shifted to M_{open}. The latter appears within the submilliseconds time domain. Such a conclusion is in line with the FTIR data [35]. We suppose that azide as well as water molecules can penetrate into the M_{open}. Whereas azide serves as a proton donor for the Schiff base, water molecules induce a decrease in p*K* of Asp-96 in the wild-type bR, its deprotonation and immediate protonation of the Schiff base. Thus, an equilibrium concentration of M_{open} (M_N) is low in the photocycle of the wild-type bR and determines the overall rate of the M decay. M_{open} (M_N) can be stabilized at high pH [20,24,27]. This phenomenon seems to be due to the fact that deprotonation of the Asp-96 is not accompanied by reprotonation of the Schiff base and the proton is probably released into the external medium. An absorption maximum of M_{open} is slightly blue-shifted in comparison with the M_{closed} in the D96N photocycle [22]. At a high pH, we have found a similar blue shift of the M absorption maximum in the wild-type bR (Radionov and Kaulen, in preparation). This is probably related to stabilization of the M_{open}.

Electron crystallographic [42] and X-ray diffraction [20] data indicate that the main conformational changes take place during the $M_1 \rightarrow M_2$ transition. Really, the inhibitors used [17,18,22] and hydrostatic pressure [53] slow down the rate of the M formation but do not affect the flash-induced M amplitude in the wild-type bR. The latter is probably due to irreversibility of the $M_1 \rightarrow M_2$ transition. However, EPR spectroscopic studies using spin labels [43,54,55] and flash-induced light scattering changes in the purple membrane suspension [56,57] indicate that some conformational changes also occur during the $M \rightarrow N$ transition. In addition, the hydrostatic pressure strongly inhibits the M decay. An ultraviolet resonance Raman study [58] has revealed a hydrophobic to hydrophilic change in the environment of Trp-182, which is localized between retinal and the cytoplasmic surface. The change in question correlates with the $M \rightarrow N$ transition. All these effects may be explained if we assume that the M_{open} and M_2 are of different conformations, M_{open} being of a larger volume than M_2. Obviously, these conformational changes leading to the penetration of a few small molecules into the protein might be rather small and occur mainly close to the surface. Subramaniam et al. [42] observed small structural changes between intermediates trapped at early and late times in the photocycle as well as between M intermediates in the wild-type bR and D96N mutant. It is not excluded, however, that conformational changes occur mainly in the direction perpendicular to the membrane plane. Note that resolution of the electron diffraction analysis in this direction is lower than in the parallel direction [59].

It is noteworthy that a high pressure decreases the maximal amplitude of the M intermediate in the D96N mutant and slightly accelerates its decay [53]. These data are also in line with our model (Fig. 2). The pronounced effect of the hydrostatic pressure on the M amplitude is due to the high concentration of the M_{open} in the D96N photocycle and possibly due to the existence of the $M_1 \leftrightarrow M_2$ equilibrium. Some acceleration of the M decay is associated with the fact that the M decay in the D96N mutant photocycle in the absence of azide is mainly associated with the reversion of the photocycle and proton uptake from the extracellular surface [60].

It is interesting that water molecules somehow participate both in the $M_2 \rightarrow M_N$ and in the $M_1 \rightarrow M_2$ transitions. We have analyzed data given in [61], namely dependence of the logarithm of the M decay rate on the relative humidity, and revealed two well-resolved components indicating to co-existing of two different mechanisms of the M decay inhibition. We suppose that a relative humidity decrease from 100% to 60–70% mainly inhibits the M decay, shifting the equilibrium between M_2 and M_{open}. Glycerol induces the same effect [17,18,62]. The $M_1 \rightarrow M_2$ transition is retarded at a lower humidity ($< 60\%$) [20,24,27].

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