Complicated character of the M decay pH dependence in the D96N mutant is due to the two pathways of the M conversion

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Abstract At high ionic strength, the pH dependence of the M intermediate decay in a photocycle of the D96N mutant bacteriorhodopsin shows a complicated behavior which is found to be due to the coexistence of two pathways of the M conversion. The M decay which dominates at pH < 5 is coupled to the proton uptake from the cytoplasmic surface and proceeds probably through the N intermediate. This pathway is inhibited by glutaraldehyde, the potent inhibitor of M decay in the wild-type bacteriorhodopsin and of the azide-facilitated M decay in the D96N mutant. Another pathway of the M decay is predominant at pH > 5. This pathway is insensitive to glutaraldehyde and some other similar inhibitors (lutetium ions, sucrose and glycerol). On the other hand, it is sensitive to the pK changes of the group X (Glu-204) in the outward proton pathway. Possibly, the M decay through this pathway represents a reverse H^+ transport process (the proton uptake from the external surface) and proceeds via the L intermediate.

Key words: Bacteriorhodopsin; Photocycle; Proton transport; Azide; Purple membrane; D96N mutant; *Halobacterium salinarium*

1. Introduction

Bacteriorhodopsin's (bR) function as a proton pump (see reviews [1–3]) is based on the light-induced deprotonation of the Schiff base, which is accompanied by formation of a short wavelength M intermediate. The proton removed from the Schiff base is released on the external surface of the purple membrane. On the other hand, the proton from the cytoplasmic surface takes part in the reprotonation of the Schiff base. The proton uptake comprises the following two major phases: (i) the proton from Asp-96 is transferred to the Schiff base and this can be monitored as the M intermediate decay; (ii) the proton from the water phase reprotonates Asp-96. This mechanism accounts for the absence of dependence of the M intermediate decay on the pH level in bulk water phase in the wild-type bR and the appearance of such dependence in the D96N mutant [4–7]. In the latter case, however, the pH dependence of the M decay rate has a complicated behavior and its slope in the logarithmic coordinates is not equal to 1 as it would have been if the reprotonation rate had been determined by the proton concentration in the water phase. Miller and Oesterhelt [8] explained these relationships assuming a complicated dependence of proton concentration in a surface layer on pH in the bulk phase. This effect, in turn, is due to the pH dependence of the surface potential. In the present study we have come to another conclusion.

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It is well known that azide and some other small weak acids accelerate the M decay in the D96N mutant, probably due to their ability to serve as penetrating proton donors [6,7,9–13]. We found that some agents (glutaraldehyde, lutetium ions, glycerol and sucrose) strongly inhibit the azide-facilitated reprotonation of the Schiff base [14]. On the other hand, these agents did not inhibit the rate of the M decay in the absence of azide at pH > 5. We have studied the pH dependence of the glutaraldehyde effect on the M decay in detail and found that glutaraldehyde does inhibit the M decay but only at low pH values. The existence of two different pathways of the M decay in the D96N mutant was postulated. The M decay in the mutant is coupled to proton uptake from both cytoplasmic and external purple membrane surfaces. The contribution of these two pathways depends on pH and this leads to the complicated character of the pH dependence of the M decay.

2. Materials and methods

All the experiments were carried out using freshly prepared purple membrane sheets from the *Halobacterium salinarium* mutant strain D96N, which was kindly donated by Prof. D. Oesterhelt (Max-Planck Institute of Biochemistry, Germany).

The measurements were performed on light-adapted purple membrane suspensions at 20°C. The bR photocycle was monitored with a single beam spectrophotometer [10,11]. Photoexcitation of bR was carried out with a YG-481 Quantel neodymium laser ($\lambda = 532$ nm; pulse half-width, 15 ns; energy, 10 mJ).

Maximal glutaraldehyde inhibition of the bR photocycle was achieved by the overnight treatment of the purple membranes with 5 mM phosphate-Tris-borate buffer, pH 8.5, containing 1% glutaraldehyde at 20°C [14]. Succinic anhydride treatment was performed according to [15].

All solutions were adjusted with a glass-electrode calibrated in H_2O-containing buffers. To determine the pD values of the deuterated solutions, values measured with the glass electrode were increased by 0.4 pD units [16].

The transition between neutral and alkaline bR forms was monitored by measuring rates of the M intermediate formation as described earlier [17].

3. Results and discussion

To minimize surface potential effects, all experiments were performed at high ionic strength (1 M NaCl). Nevertheless, the pH dependence of the M decay in the D96N showed a complicated character (Fig. 1). At pH < 3.5 and 5.5 < pH < 8, the slope was close to 1. At pH > 8 and 4 < pH < 5.5, the M decay was practically pH-independent. Glutaraldehyde treatment led to some acceleration of the M decay at pH > 5 and to deceleration at pH < 5. As a result, the pH-independent region was observed at 2.5 < pH < 5.5. Note that glutaraldehyde treatment resulted in strong inhibition of both the M decay in the wild-type bacteriorhodopsin and the azide-facili-

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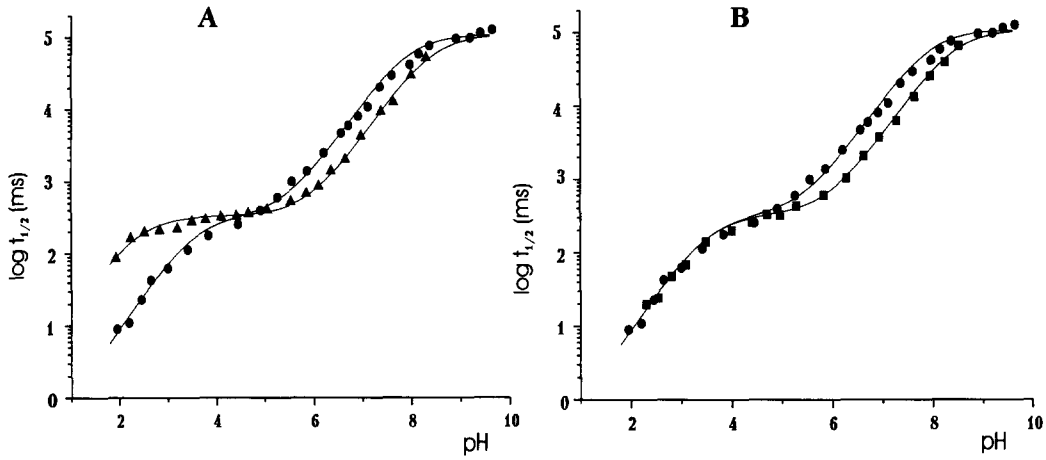

Fig. 1. Effects of glutaraldehyde (A) and succinic anhydride (B) on the pH dependence of the D96N M decay. The assay medium was 1 M NaCl, 5 mM Na-citrate-MES-phosphate-TRIS-borate buffer, 10 μM D96N bR. (●), untreated D96N bR; (▲), glutaraldehyde-pretreated D96N bR; (■), succinic anhydride-pretreated D96N bR. All theoretical curves were drawn using equation $t_{1/2} = \ln(2)/(k_1 \cdot \exp(-pH) + k_2 \cdot (1 - 1/(\exp(pK - pH) + 1)) + k_3)$. See text for details. (●), $k_1 = 7600\ c^{-1}\ M^{-1}$, $k_2 = 2.1\ c^{-1}$, $k_3 = 0.0062\ c^{-1}$, pK = 5.4; (▲), $k_1 = 480\ c^{-1}\ M^{-1}$, pK = 5.9, k_2 and k_3 are the same as for untreated bR; (■), pK = 5.9, k_1, k_2 and k_3 are the same as for untreated bR.

tated M decay in the D96N mutant [14,18–20], and, therefore, the action of glutaraldehyde on the M decay in the absence of azide resembled this inhibitory effect at pH < 5 but not at pH > 5.

Probably, the glutaraldehyde-induced acceleration of the M decay at pH > 5 is not related to its linking action. The monofunctional agent succinic anhydride induced the same acceleration of the M decay, but at the same time it did not affect the M decay at pH < 5. Moreover, succinic anhydride treatment did not affect the M decay in the wild-type bacteriorhodopsin and the azide-facilitated M decay in the D96N mutant and fully prevented the inhibitory action of glutaraldehyde (data not shown). These data point to the existence of two pathways of the M decay, differing in pH dependence.

Fig. 2 demonstrates the effect of D_2O on the M decay in the D96N mutant. It is seen that D_2O induces deceleration of the M decay at pH < 6, and slight acceleration at high pH. These data are consistent with the assumption that mechanisms of the M decay at low and high pH are different.

Fig. 3A shows the pH dependence of the single flash M magnitude. pH dependence could be fitted fairly well by the sum of two components describing titration of two groups with pK 2.35 and 5.4. Glutaraldehyde and succinic anhydride treatments induced an increase in both pK values. Note that succinic anhydride did not affect the M decay at pH < 5 whereas glutaraldehyde induced its deceleration (Fig. 1). On the other hand, both glutaraldehyde and succinic anhydride induced comparable acceleration of the M decay at pH > 5.

Fig. 3B demonstrates the interconversion of the neutral and alkaline bacteriorhodopsin forms which took place at high pH where the M decay slightly depended on pH. The M formation rates for these forms differ more than 20-fold. Transition between these forms is described fairly well by titration of a group of pK 8.6.

Fig. 4 summarizes our ideas concerning the mechanism of the M decay in the mutant protein. The experimental points of the pH dependence of the M decay fit fairly well to the theoretical curve for the rate constant (k) of the M decay (Fig. 1) which is the sum of three components (Fig. 4A):

$$k = k_1 \cdot \exp(-pH) + k_2 \cdot (1 - 1/(\exp(pK - pH) + 1)) + k_3$$

The first component is directly proportional to the proton concentration in the water phase throughout the whole pH region. The second component represents the dependence of the M decay rate on the protonation state of a group of pK 5.4. As to the third component, it is pH-independent.

The similarity of the inhibitory action of several agents on the M decay in the wild-type bacteriorhodopsin and the azide-facilitated M decay in the D96N mutant [14,20] can be accounted for by the bR photocycle scheme shown in Fig. 4B. We suppose that there is an equilibrium between two forms of the M intermediate, differing in the state of the inward proton pathway. In M_{open} a cleft permeable for water molecules as well as for small weak acids (azide) is formed in the cytosol-facing part of bR [10,14,21]. In the wild-type bacteriorhodopsin, the appearance of water near Asp-96 leads to a decrease

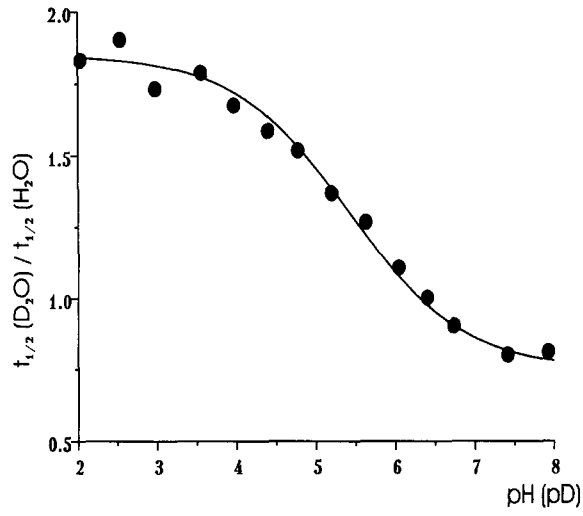

Fig. 2. Effect of D_2O on the D96N M decay. The assay medium was 1 M NaCl, 10 μM D96N bR. The solid line is drawn according to the equation: 1.85−1.1/(exp(−0.6 pH+3.25)+1).

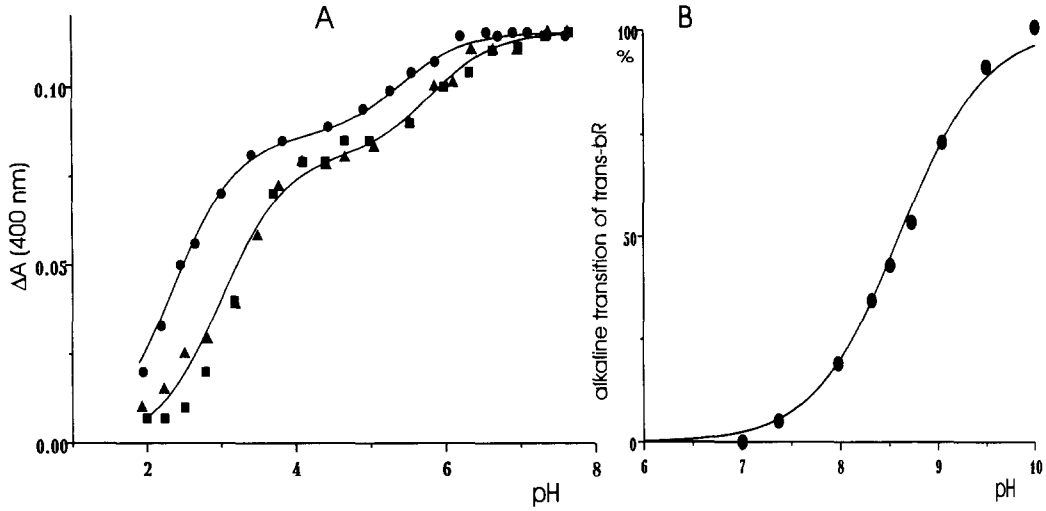

Fig. 3. pH dependence of the M magnitude measured in response to the single flash (A) and of the transition between neutral and alkaline forms of bR (B). (●), Untreated D96N bR; (▲), glutaraldehyde-pretreated D96N bR; (■), succinic anhydride-pretreated D96N bR. The assay medium was 1 M NaCl, 5 mM Na-citrate-MES-phosphate-TRIS-borate buffer, 10 μM D96N bR. A: Theoretical curves represent the sum of two curves describing titration of groups with pK 2.35 and 5.4 and a magnitude ratio 75:25 for untreated bR, and with pK 3 and 5.8 and a magnitude ratio 70:30 for treated bR. B: Theoretical curve represents titration of the group of pK 8.6.

in its pK [22–25]. In the D96N mutant, azide penetrates through this cleft and protonates the Schiff base [14,20]. This scheme is in line with the current knowledge on the light-induced bacteriorhodopsin conformational changes and its function [22–26].

We suppose that the first component (Fig. 4A) is due to the reaction associated with the M decay into the bacteriorhodopsin ground state via the M_{open} and N intermediates. During this process, protons penetrate to the Schiff base through the cleft in the M_{open} from the cytoplasmic surface of the membrane. That is why this process is sensitive to glutaraldehyde. According to our data glutaraldehyde, lutetium ions, glycerol and sucrose inhibit the azide-facilitated M decay shifting the equilibrium between two M states toward the M_{close} [14,20]. Unfortunately, it is impossible to use agents other than glutaraldehyde to confirm this conclusion. It is known that lutetium ions strongly inhibit the M decay both in the wild-type bacteriorhodopsin and in the D96N mutant in the presence of azide only at pH > 7 [19]. Glycerol does induce deceleration of the M decay at low pH, but interpretation of these data is not so obvious due to the uncertainty of the pH measurements at high concentration of glycerol or sucrose.

The second and the third components of the M decay are probably related to another process, namely, the back reaction of the photocycle. The second component becomes quite obvious when the first process is inhibited by glutaraldehyde (Fig. 1A). Both the glutaraldehyde and succinic anhydride treatments induce an alkaline shift of the pK of the second process. Perhaps, this back process represents a direct L → bR ground state transition (Fig. 4B). The back reaction dominates in the M decay at pH > 5. In line with this reasoning, the L intermediate is shown to give the bR ground state with no M state formed when the acidic (blue) bR is illuminated [27,28]. A similar process is inherent in the neutral (purple)

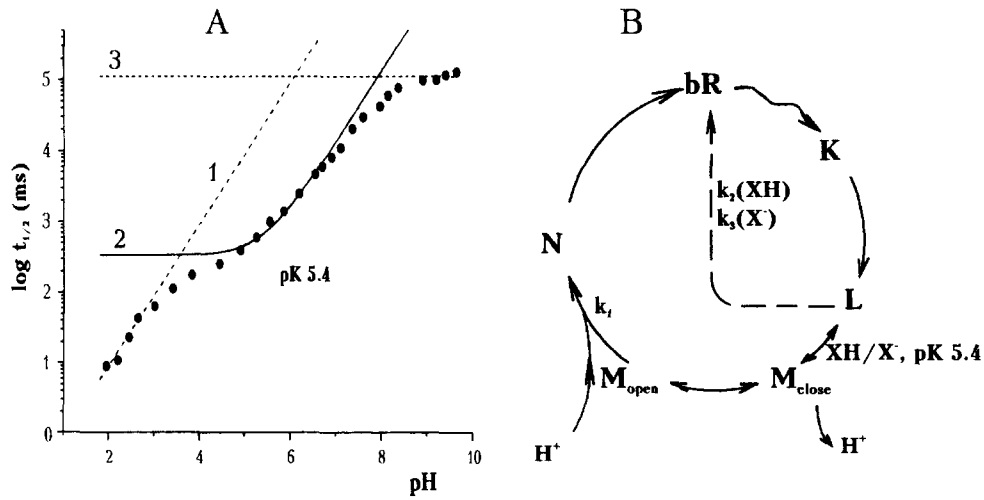

Fig. 4. A: Three components of the pH dependence of the M decay in untreated D96N bR (●). Three theoretical curves are shown, i.e. curve 1, $k_1 \cdot \exp(-pH)$; curve 2, $k_2 \cdot (1-1/(\exp(pK-pH)+1))$; curve 3, k_3. See text for details. $k_1 = 7600\ c^{-1}\ M^{-1}$, $k_2 = 2.1\ c^{-1}$, $k_3 = 0.0062\ c^{-1}$, pK = 5.4. The sum of these three components corresponds to the solid curve for untreated bR in Fig. 1. B: Two pathways of M decay in the photocycle of D96N bR.

bR at low temperature [29]. According to our data (not shown), the L decay in the photocycle of the blue acidic form of the D96N mutant is delayed no more than 4-fold in comparison with the wild-type bR. Moreover, the L to M transition is reversible in the D96N mutant at least at pH < 6 [23,30]. The L↔M equilibration is pH-dependent. It depends on the protonation state of the group X in the outward proton pathway [30]. Recently this group was identified as Glu-204 [31]. The pH-dependent L↔M equilibration accounts for the pH-dependent component of pK 5.4 in the D96N mutant (Fig. 3A; see also [30]). The second pH-dependent component of pK 2.35 is due to the transition of the neutral purple and the acidic blue bR forms, i.e. protonation of Asp-85. Thus, we suppose that the second component of the pH dependence of the M decay is associated with the proton uptake from the external surface of the purple membrane. The rate of this process is determined by the protonation state of Glu-204 which is involved in the outward proton pathway. The component in question has the same pK as the corresponding component of the pH dependence of the M amplitude (compare Figs. 1 and 3A). Glutaraldehyde and succinic anhydride induce a similar shift of the pK of both dependences (see the same figures). The mechanism of this shift is not clear. Both agents can induce small (about 1 pH unit) acidic shifts of pK of modified Lys residues. Thus, either the initial pK of this Lys residue localized near the outward proton pathway is very low or the pK shift of Glu-204 is related to some conformational changes near the proton pathway. Glutaraldehyde, lutetium ions, sucrose and glycerol influence only the equilibrium between the two forms of the M intermediates. Thus, these agents do not affect the M decay into the bR ground state via the L intermediate.

We suppose that saturation of the pH dependence of the M decay is due to the third (pH-independent) component (Fig. 3A). Probably, it is determined by the intrinsic rate of the 13-*cis-trans* isomerization taking place during the L to bR ground state transition in these protein molecules which have deprotonated groups Glu-204 and Asp-85. The rate of the 13-*cis-trans* isomerization for the second component is much higher than that of the third component because of the fact that Glu-204 is mainly in its protonated state. Isomerization seems to be catalyzed by the protonated form of Asp-85 which, in turn, is protonated by Glu-204. It is known that protonation of Asp-85 is required for *cis-trans* isomerization during dark adaptation [32,33]. If Glu-204 is deprotonated in the alkaline bR form ground state, then the L intermediate appearing during the alkaline bR photocycle should have deprotonated Asp-85 and Glu-204 just as the L intermediate formed from neutral bR at pH higher than the pK of Glu-204 in the excited state (pK=5.4). This is why the pH dependence of the M decay is insensitive to the neutral-alkaline transition.

Obviously, both the first and the second components may have deviations from linearity due to possible pH dependence of the surface potential especially at low ionic strength. Nevertheless, the complicated character of the pH dependence of the M decay at high ionic strength is mainly due to the existence of at least two different pathways of the M decay with different pH dependences. This feature should be taken into account particularly in double mutants, when one of the mutations is at Asp-96.

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