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Two bacteriorhodopsin M intermediates differing in accesibility of the Schiff base for azide

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Abstract **Glutaraldehyde treatment leads to the inhibition (i) of the M intermediate decay in wild-type bacteriorhodopsin (bR) and (ii) of the azide-facilitated M decay in the D96N mutant bR. $LuCl_3$ is shown to be a more potent inhibitor of both processes. Glycerol and sucrose are also inhibitors. None of these agents change the linearity of the azide concentration dependency of the M decay in the D96N mutant but they do shift this dependency to higher azide concentrations. It is concluded that the two M forms are in equilibrium. These M forms differ in the accessibility of the Schiff base for azide and, probably, also for water molecules. The above-mentioned agents shift the equilibrium toward the less accessible M form. The data obtained are in line with the model of azide action as the penetrating proton donor and can hardly be realized within the framework of the model of Le Coutre et al. [(1995) Proc. Natl. Acad. Sci. USA 92, 4962–4966] which assumes that a bound anionic form of azide catalyzes proton transfer to the Schiff base.**

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

1. Introduction

The light-driven proton transport in bR is coupled to the cycle of photochemical conversions via the intermediates J, K, L, M, N, and O [1–3]. It is well known now that the M intermediate, in fact, comprises a set of different forms with similar absorption spectra [4–9]. There are many pieces of evidence for protein conformational changes accompanying the proton transport, especially the reprotonation of the Schiff base by the internal proton donor D96, i.e. the decay of the M intermediate [9–14]. The mutation leading to the lack of proton donor (D96N) causes a dramatic retardation of the M decay [15,16], and this kinetic defect can be repaired by azide and other anions of small weak acids [7,17–22]. It was initially supposed that the action of azide was associated with its ability to serve as the penetrating proton donor [7,17-21], however, another model has recently been put forward [22] according to which the tightly bound anionic form of azide can catalyze proton transfer to the Schiff base.

In the present paper, we have studied the effects of different agents, which are known to inhibit the M decay in wild-type bR, on azide-facilitated M decay in the D96N mutant. The data obtained demonstrate a similarity between the inhibitory action of these agents on the wild-type and D96N bR samples. It should be stressed that the inhibitory mechanisms of the above agents are probably quite different, i.e. arrest of the conformational changes in the case of glutaraldehyde or $LuCl_3$ [23,24] and a decrease in the water activity in the case of glycerol and sucrose [20]. The data obtained are in line with the model of azide action as the penetrating proton donor. They revealed two M forms differing in the accessibility of the Schiff base for azide and, possibly, water molecules.

2. Material and methods

All the experiments were carried out using freshly prepared purple membrane sheets from the halobacterial wild-type ET1001 and mutant strain D96N. The latter was kindly donated by Prof. D. Oesterhelt (Max-Planck Institut für Biochimie, Germany).

The measurement were performed on light-adapted purple membrane suspensions at 20°C. The bR photocycle was monitored using a single-beam spectrophotometer [7,19,24]. Photoexcitation of bR was carried out with a YG-481 Quantel neodymium laser (λ = 532 nm; pulse half-width, 15 ns; energy, 10 mJ).

Maximal glutaraldehyde inhibition of the bR photocycle was achieved by treatment of purple membranes in 1% glutaraldehyde overnight at 20°C in 5 mM Na-citrate-phosphate-Tris buffer at pH 8.

3. Results.

Fig. 1A (●) shows the azide-concentration dependency of the D96N M intermediate decay which is described fairly well by the equation $k = k_1 + k_2[HN_3]$, where k_1 is the apparent first-order constant characterizing the M decay at a definite pH value in the absence of azide, and k_2 is the apparent second-order constant characterizing reprotonation of the M intermediate by the protonated form of azide. It was shown earlier [7,25] that deviation of the experimental data from the theoretical curve at high azide concentration has an apparent character, and good agreement between the data and the curve can be achieved by proper kinetic analysis.

Glutaraldehyde treatment of the wild-type purple membrane leads to 25-fold deceleration of the M intermediate decay (Fig. 2), whereas the M decay in D96N bR in the absence of azide was not affected (Fig. 1A). Lutetium ions at pH > 6.5 induce 400-fold inhibition of the M decay in the wild-type bR photocycle (not shown), however, they do not influence the M decay in the mutant bR (Fig. 1B). Glycerol and sucrose, inhibitors of the M decay of wild-type bR [20] (Fig. 3), do not affect the D96N M decay either (Fig. 1A). Thus, neither of the above-mentioned agents produces any effect on k_1. Nevertheless, all of these agents induce strong inhibition of azide-dependent reprotonation of the M intermediate (Fig. 1A,B). In all cases, the same rate of M decay in inhibitor-treated preparations can be achieved by increasing the azide concentration, and all the dependencies are shifted toward the range of higher azide concentration (Fig. 1A,B).

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Abbreviations: bR, bacteriorhodopsin

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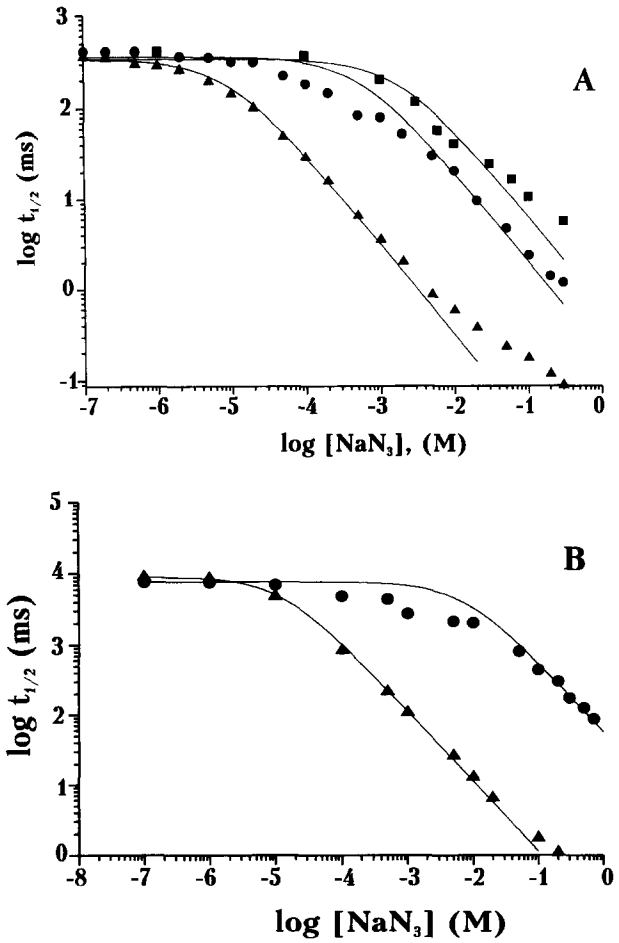

Fig. 1. Dependence of the M decay of D96N bR on azide concentration. (A) Assay medium (▲,●): 1 M NaCl, pH 5, 10 μM D96N bR. (▲) No addition, (●) glutaraldehyde-pretreated D96N bR, (■) untreated D96N bR in 83% glycerol (w/w). All theoretical curves were drawn using the equation $t_{1/2} = \ln(2)/(k_1 + k_2[HN_3]) = \ln(2)/(k_1 + k_2k_3[NaN_3])$. See text for details. (▲) $k_1 = 2\ s^{-1}$, $k_2k_3 = 220\,000\ s^{-1}\ M^{-1}$, (●) $k_1 = 1.9\ s^{-1}$, $k_2k_3 = 3500\ s^{-1}\ M^{-1}$, (■) $k_1 = 2\ s^{-1}$, $k_2k_3 = 1120\ s^{-1}\ M^{-1}$. (B) Assay medium: 1 M NaCl, 50 mM HEPES, pH 7, 10 μM D96N bR. (▲) No addition, (●) 2 mM $Lu(Cl)_3$ was added. Theoretical curves were drawn according to equation given in (A). (▲) $k_1 = 0.08\ s^{-1}$, $k_2k_3 = 6200\ s^{-1}\ M^{-1}$, (●) $k_1 = 0.091\ s^{-1}$, $k_2k_3 = 12.4\ s^{-1}\ M^{-1}$.

Thus, these agents all lead to a decrease in k_2. Glutaraldehyde treatment decreases k_2 by a factor of 60 (Fig. 1A). An increase in the glycerol content in the incubation medium up to 83% (w/w) leads to a 200-fold decrease in k_2 (Fig. 1A). In the presence of Lu^{3+}, k_2 is lowered by a factor of 500 (Fig. 1B). Just as in the wild-type protein, the inhibitory effect of Lu^{3+} is especially pronounced at pH > 6, and inhibition is fully reversed after addition of EDTA (not shown).

Fig. 3 demonstrates the qualitative similarity between the effects of sucrose and glycerol on the M decay in wild-type bR and in the D96N mutant in the presence of azide. It should be mentioned that Cao et. al. [20] did not observe any effect of sucrose on the M decay in D96N bR in the presence of azide. The reason for this discrepancy is unknown.

Azide is known to accelerate the M decay not only in the D96N mutant but also in the wild-type bR [19,22]. The dependence of the M decay on azide concentration can be fitted well by the same equation as in the case of D96N bR (Fig. 2). The main difference is in the absolute k_2 value. For the wild-type protein, the k_2 value is 250-fold lower than for the mutant. Glutaraldehyde treatment of the wild-type protein induces a 4-fold decrease in k_2.

4. Discussion

The data obtained show that strong inhibition of azide-dependent M decay in D96N bR can be induced by glutaraldehyde treatment, and by addition of $LuCl_3$, as well as by an increased content of glycerol and/or sucrose. In all cases, inhibition is accounted for by the decrease in the rate constant characterizing the second-order process of azide interaction with bR (k_2). One can conclude that the mechanism of inhibition is based on the existence of steric hindrance against azide diffusion. However, such a reason cannot provide an exhaustive explanation for all the phenomena.

First of all, we would like to emphasize that the effects of different agents on the D96N M decay in the presence of azide correlate with those on the M decay in wild-type bR without azide. In other words, the effects on the process of Schiff base reprotonation by the external proton donor (azide) and by the internal proton donor (D96) proved to be similar. There is a qualitative similarity between the inhibition of the two processes by increasing content of glycerol and/or sucrose (Fig. 3). $LuCl_3$ (at pH > 6.5) induced deceleration of the M decay in the wild-type bR which became more than 20-fold slower than in glutaraldehyde-pretreated bR (not shown, see also [24]). $LuCl_3$ was also revealed to be a more potent inhibitor of azide-dependent M decay in D96N bR as compared with glutaraldehyde.

Note that the mechanism of inhibition by the various agents used may be different. There is currently much evidence for the existence of protein conformational changes associated with the bR proton-transport photocycle. These data are based on light-scattering phenomena in the purple membrane suspensions [10,11], electron diffraction data [12,13], the effect of hydrostatic pressure on the photocycle [9], and study of spin-labeled bR [14]. Inhibition by glutaraldehyde and $LuCl_3$ possibly results from the restriction of the

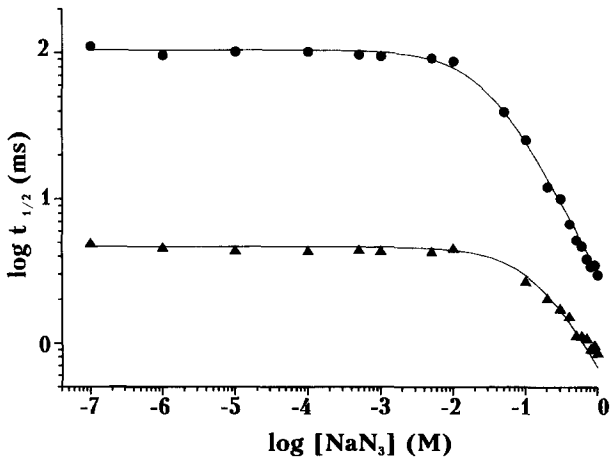

Fig. 2. Dependence of the M decay of wild-type bR (▲) and glutaraldehyde-pretreated wild-type bR (●) on azide concentration. Assay medium: 1 M NaCl, pH 5, 10 μM bR. Theoretical curves were drawn according to equation given in the legend to Fig. 1A. (▲) $k_1 = 150\ s^{-1}$, $k_2k_3 = 900\ s^{-1}\ M^{-1}$, (●) $k_1 = 6.7\ s^{-1}$, $k_2k_3 = 227\ s^{-1}\ M^{-1}$.

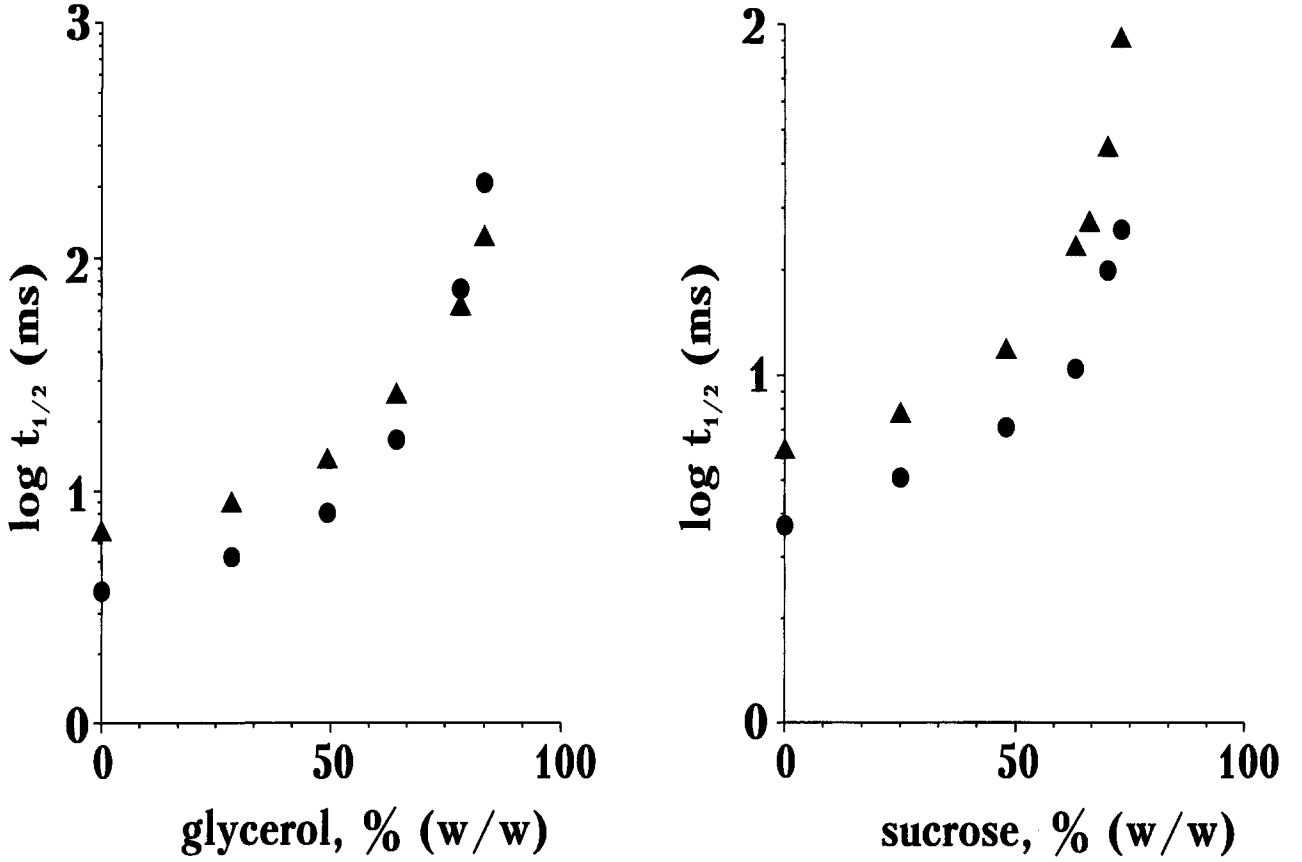

Fig. 3. Dependence of the M decay of wild-type bR (▲) and D96N bR (●) in the presence of NaN_3 (1 mM) on glycerol and sucrose contents in the incubation medium. Assay medium: 50 mM Na-citrate, pH 5, 10 μM bR.

conformational flexibility of the protein. On the other hand, the effect of glycerol and sucrose seems to be due to a decrease in the water activity [20]. Our model of the bR photocycle along with the explanation of the observed inhibitory phenomena is shown in Fig. 4. Some years ago we supposed the appearance of a water cleft between the cytoplasm and D96 during the bR photocycle. The cleft was assumed to facilitate the protonation of D96 [19,26]. Some papers from Lanyi's laboratory [9,27–29] have recently appeared in which a similar scheme was worked out. The authors concluded that the water channel has an additional function: water molecules lead to a decrease in the pK of D96 and thus facilitate proton transfer from D96 to the Schiff base. Recently, the formation of a cleft was directly proved in structured studies carried out by the Henderson group [13].

We suppose (Fig. 4) the existence of two M forms, i.e. 'closed' and 'open', differing in the presence of the water cleft between the cytoplasm and D96 (and, possibly, the Schiff base). It is noteworthy that there is a fast equilibration between these two forms. Whereas the appearance of the water cleft facilitates proton exchange between D96 and the Schiff base in the wild-type bR, the cleft in the mutant form increases the permeability of azide. The only difference between the kinetic schemes for the wild-type and mutant bR is the following: wild-type bR has a fixed ratio between the proton donor and acceptor but this ratio is easily changeable in the mutant. Within the framework of the scheme, the equation describing the dependence of the M decay rate on the azide concentration transforms as follows: $k = k_1 + k_2[HN_3] = k_1 + k_{11}k_{22}[HN_3]/(k_{11} + k_{-11})$, when $k_{11} < k_{22}[HN_3]$. All the agents under consideration may affect the equilibrium between M_{closed} and M_{open}, shifting it toward M_{closed}, possibly due to the stabilization of M_{closed} compared to M_{open}. The mechanisms of stabilization may be different for various agents. An equilibrium shift toward M_{closed} would induce the inhibition of the M decay at a given azide concentration without effect on the linearity of the dependence of the M decay upon the azide concentration.

The effectiveness of azide in wild-type bR is similar to that for the inhibited D96N sample. Moreover, the effect of glutaraldehyde treatment of wild-type bR is much smaller than that of the D96N mutant. We suppose that the presence of D96 in the inward proton channel greatly restricts Schiff base accessibility to azide in wild-type bR and therefore the accessibilities of the two M forms become of the same order of magnitude. In this case the direct conversion of M_{closed} into N can take place.

It should be stressed that our data are in line with the model of azide action as a penetrating proton donor and can scarcely be realized within the framework of the model of Le Coutre et. al. [22] according to which a bound anionic form of azide catalyzes proton transfer to the Schiff base. In the latter case, it is necessary to suppose that the effects of all the above agents lead to a decrease in anion binding with bR, which seems improbable.

The existence of two functionally distinct M intermediates (MI and MII) in the bR photocycle is currently a widely held point of view. It is supposed that the Schiff base is in contact

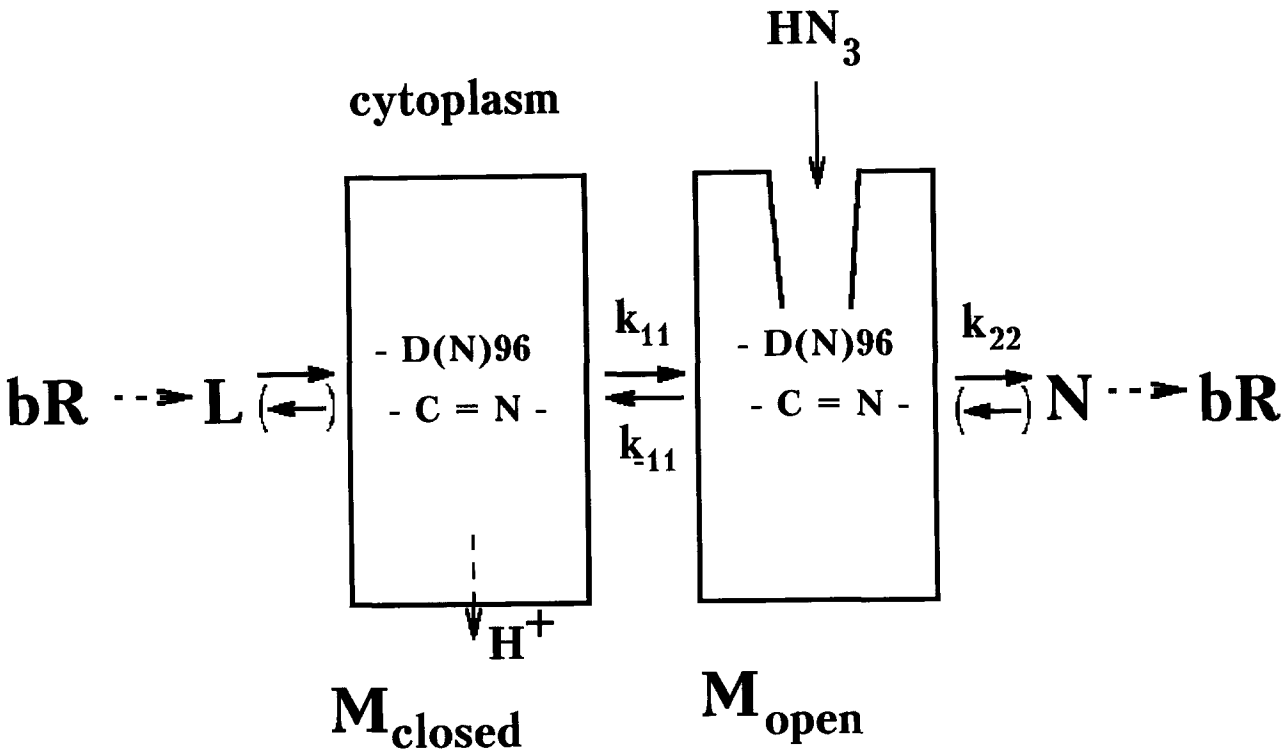

Fig. 4. A tentative scheme of the bR photocycle. See the text for details.

with the outward proton pathway in the MI state and with the inward proton pathway in the MII state. Whereas the functional difference between MI and MII (reprotonation of the Schiff base by D85 or D96) was unambiguously demonstrated at low temperature with FTIR spectroscopy [4], the kinetics characteristics of the MI→MII transition are still obscure. According to Varo and Lanyi [5], one of the components of the optical density increase within the region of M absorption is due to L↔MI equilibration, whereas the second component reflects the MI→MII transition. According to our data [7,25], these two components belong to two parallel bR photocycles. It appears that M_{open} is the most likely candidate for the role of MII, whereas M_{closed} may be MI (or one of its forms). Note that all of the agents under consideration do not affect the maximal amount of the M intermediate formed in wild-type bR but decrease this parameter in the D96N mutant by 20%. This phenomenon indicates that an irreversible L→M step must precede the $M_{closed} \leftrightarrow M_{open}$ equilibration in wild-type bR. Thus, we assume that none of the kinetic phases measured at 400 nm as the appearance of the M intermediates reflects the MI→MII transition. A different situation exists in the D96N photocycle. In this case, all the reactions between the L and M intermediates including M_{open} must be reversible. We suppose that the equilibration between M_{closed} and M_{open} proceeds on the time scale of several microseconds. This conclusion is based on results of measurement of the rate of azide interaction with the M state of D96N bR [7,25]. According to our study, the half-time of M decay in 2 M azide is equal to 20 s.

It should be mentioned that in the present work the kinetic analysis was made assuming that the M decay is described by only one exponential component. This is the case for untreated bR, whereas the M decay is clearly biphasic in inhibitor-treated bR. However, in this case the analysis also led to the same conclusions (Radionov and Kaulen, in preparation).

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