Interrelations of M-intermediates in bacteriorhodopsin photocycle

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The photocycles of the wild-type bacteriorhodopsin and the D96N mutant were investigated by the flash-photolysis technique. The M-intermediate formation (400 nm) and the L-intermediate decay (520 nm) were found to be well described by a sum of two exponents (time constants, $\tau_1 = 65$ and $\tau_2 = 250\,\mu s$) for the wild-type bR and three exponents ($\tau_1 = 55\,\mu s$, $\tau_2 = 220\,\mu s$ and $\tau_3 = 1$ ms) for the D96N mutant of bR. A component with $\tau = 1$ ms was found to be present in the photocycle of the wild-type bacteriorhodopsin as a lag-phase in the relaxation of photoresponses at 400 and 520 nm. In the presence of Lu^{3+} ions or 80% glycerol this component was clearly seen as an additional phase of M-formation. The azide effect on the D96N mutant of bR suggests that the 1-ms component is associated with an irreversible conformational change switching the Schiff base from the outward to the inward proton channel. The maximum of the difference spectrum of the 1-ms component of D96N bR is located at 404 nm as compared to 412 nm for the first two components. We suggest that this effect is a result of the alteration of the inward proton channel due to the $Asp^{96} \rightarrow Asn$ substitution. Proton release measured with pyranine in the absence of pH buffers was identical for the wild-type bR and D96N mutant and matched the $M \rightarrow M'$ conformational transition. A model for M rise in the bR photocycle is proposed.

Bacteriorhodopsin; Photocycle; M-intermediate; Protein conformational changes

1. INTRODUCTION

The M-intermediate formation is a key step in the bacteriorhodopsin (bR) photocycle associated with the Schiff base (SB) deprotonation and proton release in the bulk solution. A number of researchers have been led to consider (primarily on kinetic grounds) that there exists a more complex pathway of M-formation than that proposed before [1]. Some of them suggest two kinetically different M forms arising from two different L forms [2,3]; others postulate an equilibrium $L \rightleftharpoons M$ and irreversible $M \rightarrow M'$ step as an explanation of heterogeneity of M [4]. In the present paper we report observations indicating the branching pathway of the formation of two kinetically different M-intermediates including the irreversible $M \rightarrow M'$ conformational transition for both M-forms. Proton release in the bulk solution in the absence of buffer is suggested to accompany this conformational change.

2. MATERIALS AND METHODS

In all experiments, purple sheets from the *Halobacterium halobium* 1001 strain were used. The methods for preparing the purple membrane (pm), for measuring photocycle intermediates and pH changes were described elsewhere [5]. Pyranine was employed as a pH indicator. All measurements were carried out at 20°C. The time-resolved

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Abbreviations: bR, bacteriorhodopsin; SB, Schiff base; pm, purple membrane; EDTA, ethylenediaminetetraacetate.

absorbance change curves were decomposed into components with the aid of the DISCRETE program written by professor Provencher. HCl, NaOH and NaCl were of OSCh grade; NaN_3 and EDTA were from Sigma. Mutant D96N bR was kindly gifted by professor Oesterhelt.

3. RESULTS AND DISCUSSION

M-formation measured at 400 nm and L-decay at 520 nm were approximated with two components, having $\tau_1 = 65\,\mu s$ (60% of the total amplitude) and $\tau_2 = 250\,\mu s$ (40%), for the wild-type bR and with three components, having time constants 55 μs (30%), 220 μs (50%) and 1 ms (20%), for D96N bR. The 1 ms component was found to be present in the photocycle of the wild-type bR as a lag-phase in the decay of photoresponses at 400 and 520 nm, and as a component in the photoresponse at 335 nm in addition to the fast components associated with the $bR \rightarrow K$ and $K \rightarrow L$ transitions. Under conditions of 2 mM Lu^{3+} ions or 80% glycerol this component appeared in the M-formation of the wild-type bR (Fig. 1, curves 1,2). Fig. 1 shows the photoresponse at 400 nm of the wild-type bR under 40% sucrose, 2 mM $LuCl_3$, pH 7.0 conditions (curve 5) and after the addition of 10 mM EDTA (curve 6). One can see that the component at the 40-ms time scale appears at the expense of the first two. In the case of the glycerol condition the appearance of the M-intermediate matched the K-intermediate decay at 570–680 nm for both protein types, while L-intermediate decay at 460–600 nm precedes M-formation in the water solution.

The 1-ms component differs from the 55-μs and 220-μs components in D96N bR. The maximum of the dif-
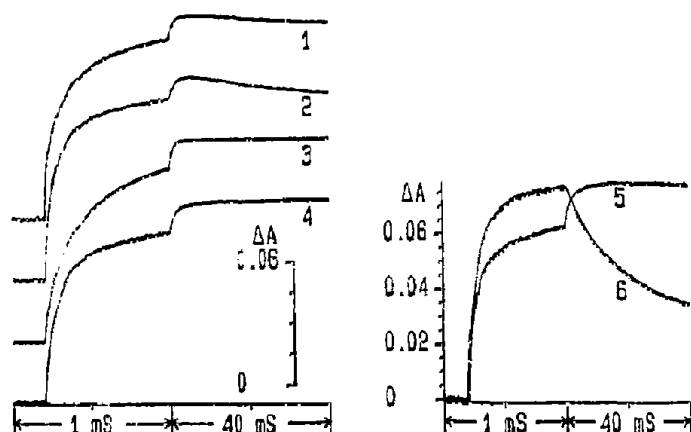
Fig. 1. Laser flash-induced absorbance changes measured at 400 nm for the wild-type bR (1, 2, 5, 6) and D96N bR (3, 4) under different conditions. 1, 2 mM LuCl₃; 2 and 4, 80% glycerol; 3, 100 mM NaCl; 5, 40% sucrose, 2 mM LuCl₃; 6, as in (5) plus 10 mM EDTA. All signals were obtained at pH 7.0.

ference spectrum of the 1-ms component is located at 404 nm (Fig. 3A) as compared to that at 412 nm for the 55- and 220-μs components. Under the D_2O condition the first two components were slowed down by a factor of 5, while the third one was slowed down by a factor of 3. Under 60% sucrose and 80% glycerol conditions the first two components were found to be faster than the same components in the water solution, while the third component was slowed down.

Azide is well-known to accelerate the M-decay of D96N bR [6]. The decay time of M was decreased from 200 ms at pH 5.0 to 1.5 ms already in the presence of 7.5 mM azide. It was found that for different kinetic models suitable for 3-exponent M-formation the best approximation was obtained when azide was suggested to protonate the SB of all M forms simultaneously (Scheme 3). This accounts for a dramatic decrease in the amplitude of the photoresponse at 400 nm (Fig. 2A). When the azide concentration was 1 M, the rate of the M-decay was 20 μs (Fig. 2B). After rapid reprotonation of M a red-shifted intermediate with a differential max-

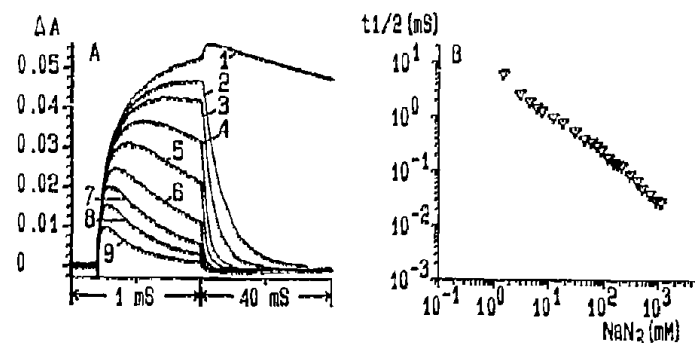Fig. 2. A: absorbance changes at 400 nm for D96N bR at pH 5.0. 1, no additions; 2–9, in the presence of 1.5, 3, 7.5, 20, 50, 150, 400 and 1100 mM NaN₃, respectively. B: the logarithmic plot for the time constant of M-decay vs. azide concentration.
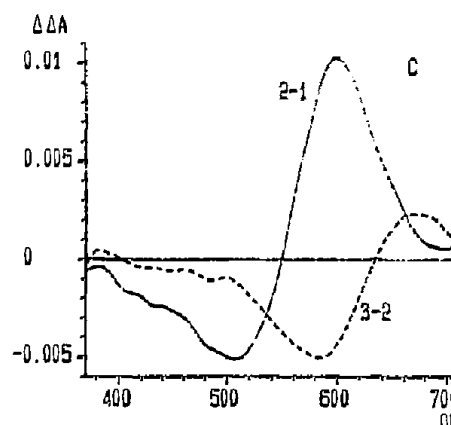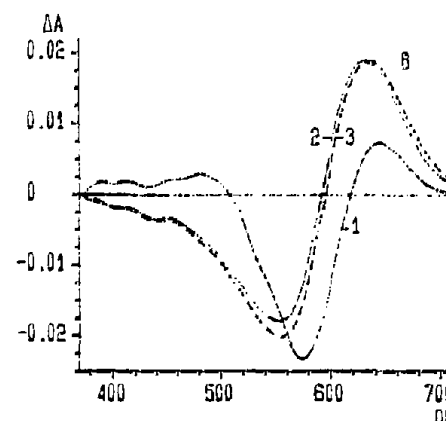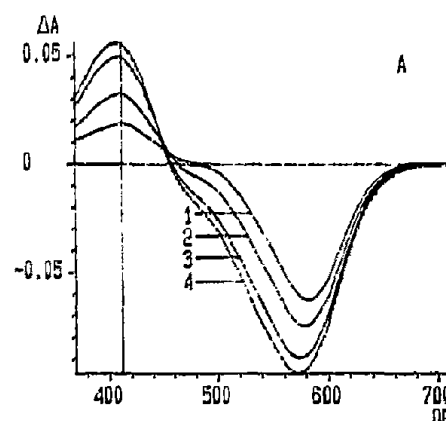

Fig. 3. A: time-resolved difference spectra for D96N bR at pH 5.0, measured after a laser flash. 1, 2, 3 and 4, 0.05, 0.25, 0.9 and 2.2 ms, respectively. B: difference spectra for D96N bR in the presence of 1 M azide, pH 5.0, measured after a laser flash. 1, 2 and 3, 0.05, 1.1 and 2.5 ms, respectively. C: spectra obtained from spectra 1–3 in B by subtracting spectrum 1 from spectrum 2 (curve 2-1) and spectrum 2 from spectrum 3 (curve 3-2).

imum at 630 nm was formed (Fig. 3B, curve 1). Under D_2O conditions when the rates of the first two components were increased from 55 and 230 μs to 250 μs and 1.6 ms, the rate of the azide effect did not change significantly. This allowed us to obtain O-like intermediate rise signals with identical rate constants as the M intermediate. The first two components of the SB deprotona-
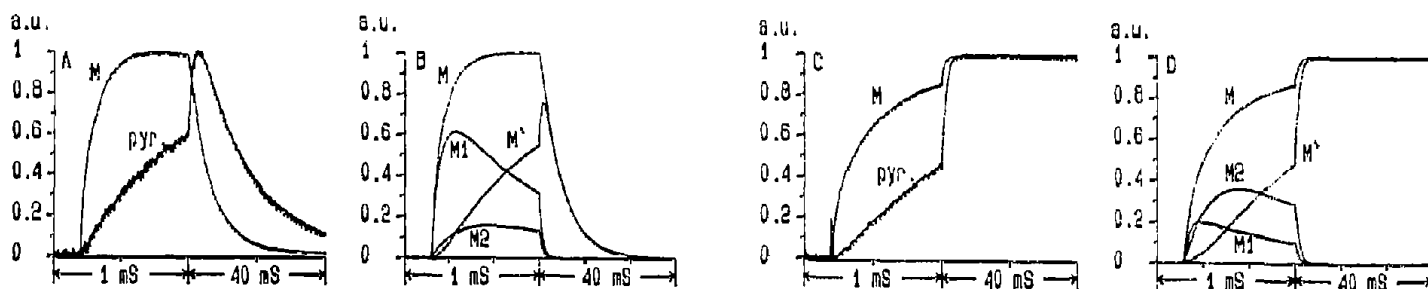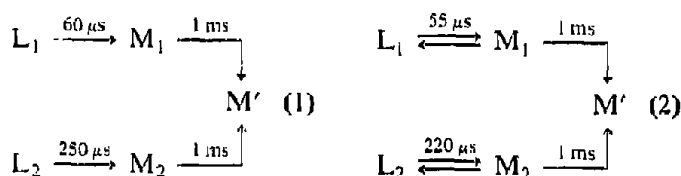
Fig. 4. The M-formation and proton release into the bulk water phase (the pyranine responses). The wild-type bR (A) and the D96N mutant of bR (C). Graphical representations for Schemes 1 and 2 are shown in B and D, respectively.

tion (L-decay) were found to be insensitive to the presence of azide, while the third component disappeared in the L-decay and appeared during direct formation of the second batho-form with a different maximum at 638 nm from the first one. The second O-like intermediate with the 3-4 ms time constant under H_2O conditions and 7-8 ms under D_2O conditions is transformed into an N-like form. This corresponds well with the M→N transition of the wild-type bR. These data indicate that D96N bR with the protonated SB (by azide) exhibits very similar transitions when M forms are replaced by O-like intermediates as compared to transitions of M-intermediates in the wild-type bR. We interpret these observations in terms of protein conformational changes. Such changes may be initiated at the early stages of the photocycle (trans-cis isomerisation, for example). The first conformational transition with time constant 1 ms reflects the SB switch from the outward proton channel, and the second one with time constant 4-5 ms may reflect the SB approach to the internal proton donor when the latter is available. Proton release in the bulk medium in the absence of buffers appears to have the same time constant as the third component of M-formation. Proton release has the same activation energy as the third component of M (not shown). Moreover, under D_2O conditions when the third component is slowed down to 3.3 ms, the rate of deuterium release in the bulk medium measured with pyranine prepared on D_2O was 3.3 ms. This may be explained with two alternatives. 1. Protein conformational transition effects changes in the membrane–water interface followed by a break in the surface proton-conducting chain and enhancing the probability of proton transfer into the bulk. 2. The probability of proton release into the bulk increases with the removal of SB from the outward proton channel with a time constant 1 ms. The second conformational transition at 4-5 ms is likely to facilitate the SB reprotonation and N formation. It is associated with the appearance of the positive phase of the light-scattering change signal in pm suspension [7]. According to our findings concerning two phases in the L-decay independent of the azide concentration we propose here a convenient model for M rise for the wild-type (1) and D96N mutant of bR (2)

$$L_1 \xrightarrow{60\,\mu s} M_1 \xrightarrow{1\,ms} \atop M'\ (1)$$

$$L_2 \xrightarrow{250\,\mu s} M_2 \xrightarrow{1\,ms}$$

$$L_1 \xrightleftharpoons{55\,\mu s} M_1 \xrightarrow{1\,ms} \atop M'\ (2)$$

$$L_2 \xrightleftharpoons{220\,\mu s} M_2 \xrightarrow{1\,ms}$$

The action of azide on D96N bR may be presented on the basis of Scheme 2 as folows (time constants for an azide concentration of 1 M were used):

$$L_1 \xrightleftharpoons{55\,\mu s} M_1 \xrightarrow{1\,ms} \quad \xrightarrow{azide,\ 20\,\mu s} O630 \xrightarrow{1\,ms}$$

$$M' \xrightarrow{azide,\ 20\,\mu s} O638 \xrightarrow{4\,ms} N \quad (3)$$

$$L_2 \xrightleftharpoons{220\,\mu s} M_2 \xrightarrow{1\,ms}$$

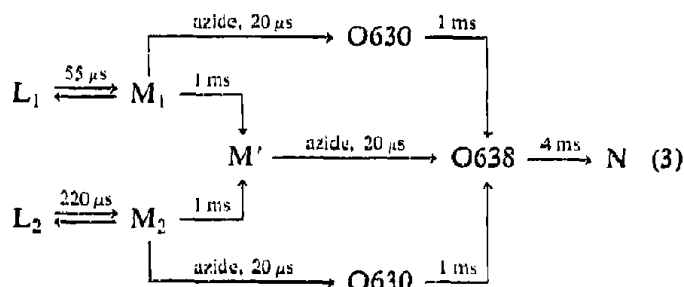$$\xrightarrow{azide,\ 20\,\mu s} O630 \xrightarrow{1\,ms}$$

Fig. 4 shows M formation and pyranine protonation in the absence of buffer for the wild-type bR and D96N bR (A and C, respectively) and the graphical representation for Schemes 1 and 2. We have suggested that glycerol or Lu^{3+} ions bring about a conformational distortion of the wild-type bR and the back M→L reaction is induced, so the 1 ms component appears in the photoresponse at 400 nm as a 'real' component, while in the photoresponse of the undistorted bR, the 1 ms component is presented as a lag-phase at 400 nm.

REFERENCES

[1] Lozier, R.H., Bogomolni, R.A. and Stoeckenius, W. (1975) Biophys. J. 15, 955-963.
[2] Butt, H.-J., Fendler, K., Der, A. and Bamberg, E. (1989) Biophys. J. 56, 851-859.
[3] Butt, H.-J., Fendler, K., Der, A., Bamberg, E., Tittor, J. and Oesterhelt, D. (1989) EMBO J. 8, 1657-1663.
[4] Varo, G. and Lanyi, J.K. (1991) Biochemistry 30, 5008-5022.
[5] Drachev, L.A., Kaulen, A.D., Skulachev, V.P. and Zorina, V.V. (1987) FEBS Lett. 226, 139-144.
[6] Tittor, J., Soel, C., Oesterhelt, D., Butt, H.-J. and Bamberg, E. (1989) EMBO J. 8, 3477-3482.
[7] Drachev, L.A., Kaulen, A.D. and Zorina, V.V. (1989) FEBS Lett. 243, 5-7.