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On the two forms of bacteriorhodopsin

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Abstract

In our previous work [(1993) FEBS Lett. 313, 248–250; (1993) Biochem. Int. 30, 461–469] M-intermediate formation of wild-type bacteriorhodopsin was shown to involve two components differing in time constants (τ_1 = 60–70 μs and τ_2 = 220–250 μs), which were suggested to reflect two independent pathways of M-intermediate formation. The contribution of the fast M was 4-times higher than the slow one. Our present research on M-intermediate formation in the D115N bacteriorhodopsin mutant revealed the same components but at a contribution ratio of 1:1. Upon lowering the pH, the slow phase of M-formation vanished at a pK of 6.2, and in the pH region 3.0–5.5 only the M-intermediate with a rise time of 60 μs was present. A 5–6 h incubation of D115N bacteriorhodopsin at pH 10.6 resulted in the irreversible transformation of 50% of the protein into a form with a difference absorbance maximum at 460 nm. This form was stable at pH 7.5 and had no photocycle, including M-intermediate formation. The remaining bacteriorhodopsin contained 100% fast M-intermediate. The disappearance of the 250-μs phase concomitant with bR460 formation indicates that at neutral pH bacteriorhodopsin exists as two spectroscopically indistinguishable forms.

Key words: Bacteriorhodopsin; Photocycle; M-Intermediate

1. Introduction

In our previous paper [1,2] the photocycles of wild-type bacteriorhodopsin (WT bR) and the D96N mutant were investigated. We have described the formation of two kinetically different M-intermediates with rise times of 55–60 and 220–250 μs. The M $\rightarrow$ M′ conformational transition, which was visible for D96N bR as an additional phase during M-formation with a time constant of 1 ms, was postulated for both M_1 and M_2 intermediates of D96N bR, as well as for WT bR. This step was shown to be associated with proton appearance in the bulk water solution and protonation of the pH indicator, pyranine, in the absence of buffer. The 10 nm shift of the minimum of the difference absorbance spectrum, corresponding to the 55–60 μs phase of M-formation as compared to the 220–250 μs phase, indicates that the 55–60 and 220–250 μs phases can be attributed to parallel or branched photocycles rather than to the sequential model with back reactions suited to multi-exponential M-formation and L-decay proposed in [3]. The data presented herein prove that the 60 and 250 μs components of M-intermediate formation reflect parallel photocycles of two spectroscopically indistinguishable bR forms at neutral pH.

The existence of two bR forms at neutral pH has already been proposed [4]. Based on resonance Raman data, the authors provided evidence for a mixture of bR(α) and bR(β). Only bR(α) was suggested to form an M-intermediate during the photocyle, with a time constant comparable to that of the first M-intermediate formation presented herein and in [1,2]. Our data obtained for D115N bR indicate that the second form of bR is able to form an M-intermediate with a time constant of 220–250 μs at pH $\geq$ 6.2.

2. Materials and methods

All experiments were carried out using freshly prepared purple membranes (pm) of WT and D115N bR from strain ET 1001. The pm preparation, photocycle registration and absorbance spectra measurements were described elsewhere [1,2,5].

The fit of the experimental curves to the sum of exponentials was obtained with the DISCRETE program [6].

The halobacterium strain mutant, D115N, was graciously donated by Prof. R. Needleman, Wayne State University School of Medicine, USA.

3. Results and discussion

M-Intermediate formation measured at 400 nm comprises two components with time constants of 60–70 and 220–250 μs for WT bR and 60–70 and 240–270 μs for D115N bR. The amplitude ratio was 4:1 for WT bR and 1:1 for D115N bR. Upon lowering the pH the second

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Abbreviations: bR, bacteriorhodopsin; WT bR, wild-type bacteriorhodopsin; SB, Schiff base; pm, purple membranes.

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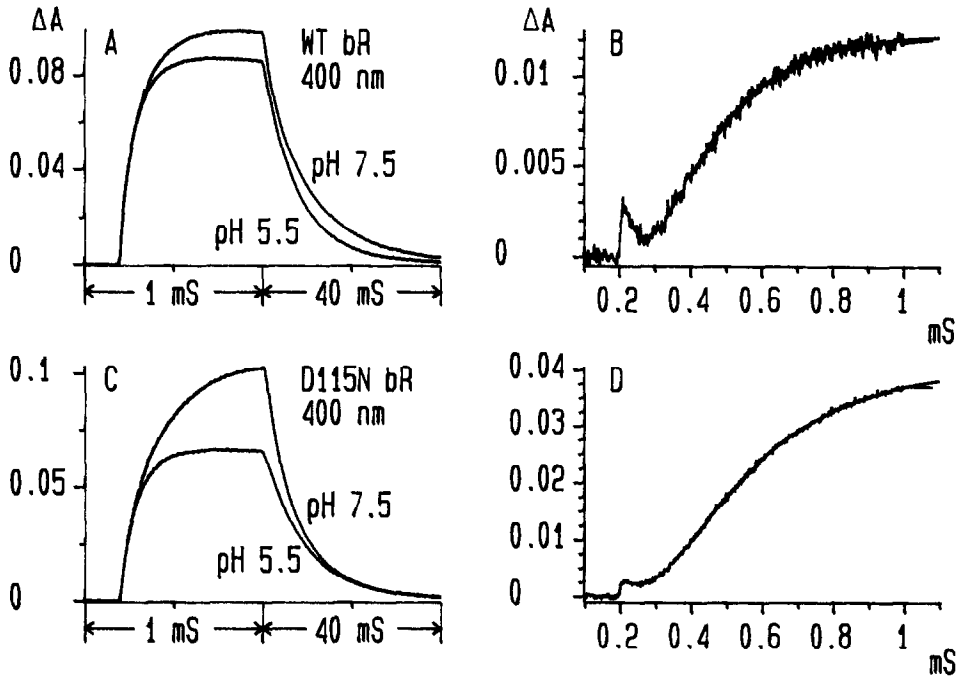

Fig. 1. M-Intermediate formation at 400 nm for WT bR (A) and D115N bR (C) at pH 7.5 and 5.5. The curves were obtained by subtracting the photosignals measured at pH 5.5 from those measured at pH 7.5 for WT bR (B) and D115N bR (D) and its approximation by the time constant of 220–240 μs.

component of the M-intermediate formation was found to vanish at a pK of 6.2. Fig. 1 shows the M-formation at 400 nm for WT bR and D115N bR at different pH values. Upon a pH transition from 7.5 to 5.5 the amplitude of the photosignal at 400 nm dropped by half for D115N bR. The amplitude decrease for WT bR made up 10–15% of the total amplitude, a value equal to the contribution of the second component in the photosignal at 400 nm (Fig. 1A,C). The distinctions between the signals measured at pH 7.5 and pH 5.5 for WT bR and D115N bR were of a complex nature, revealing a fast positive component, a lag-phase at 20–40 μs, and a component with τ = 220–250 μs (Fig. 1B,D). The first two components are likely to account for perturbations at the fast stages of the photocycle upon the pH transition, and the component with τ = 220–250 μs reflects the disappearance of the second phase of the M-rise. The pH-dependence of the photosignal amplitude comprises two pK's at 6.2 and 2.5. The pK value of 2.5 represents one of the well-known transitions of bR into the blue acid form, with the photocycle lacking SB deprotonation and M-intermediate formation. The transition with a pK of 5.8 was shown earlier in the work of Zimanyi et al. [7] for D96N and D115N/D96N bR. The authors concluded that the $M_2 \rightarrow M_1$ back reaction was pH-dependent and linked to the release of a proton from a group with a pK_a of 5.8.

Time-resolved difference absorbance spectra measured for D115N bR at pH 7.5 and 5.5 (not shown) revealed that at pH 5.5 a mixture of M- and L-intermediates existed 1 ms after a flash, while at pH 7.5 the L-intermediate transformed almost completely into the M-form. We failed to find a component for the L-intermediate decay into the ground state of bR at pH 5.5 which was different from that of the M-decay. Therefore, the L-intermediate decay apparently occurs with a time constant comparable to that of the M-intermediate decay, and the interpretation, in terms of the $M_2 \rightarrow M_1$ back reaction, is suited to this phenomenon. Nevertheless, the following experiment demonstrates that here we are dealing with two forms of bR with pK's for M-intermediate formation of 2.5 for the 60-μs component and of 6.2 for the 250-μs component.

On changing the pH from 7.5 to 10.6 a portion of the D115N protein (15–20%) transformed into the bR form with a difference maximum at 460 nm, immediately after the pH was adjusted to 10.6 (Fig. 2A). Another portion of the protein (60–70%) transformed into the bR460 form very slowly with a time constant of several hours. Lowering the pH from 10.6 to 7.5 resulted in the transformation of 50% of the protein back into bR568. The remaining portion of the protein stayed in the bR460 form, which was found to be stable for days at neutral pH. Apparently, the bR460 portion transformed into the form lacking a distinct maximum in the visible spectrum and having a diffuse maximum (or a number of maxima) in the 250–350 nm wavelength region (the absorption increase in this region is shown in Fig. 2A,B).

The bR568 $\rightarrow$ bR460 process, which takes hours at pH 10.6, can be drastically accelerated. Fig. 2A demon-

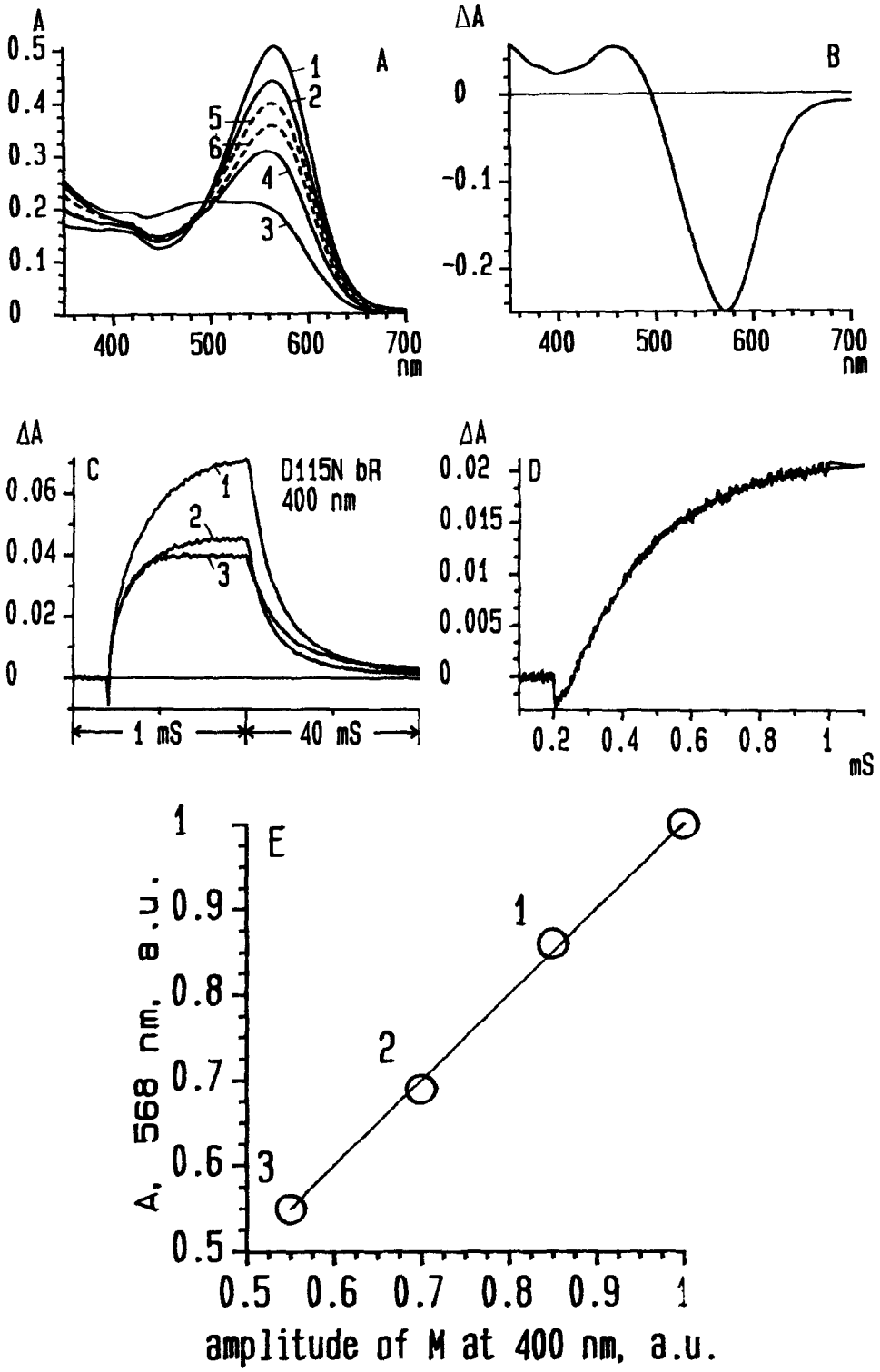

Fig. 2. (A) Absorbance spectra measured for D115N bR at pH 7.5 (curve 1), immediately after adjustment to pH 10.6 (curve 2). Spectra measured at pH 10.6 (curve 3) and 7.5 (curve 4) after a 5-h incubation at pH 10.6. Curves 5,6, spectra measured at pH 7.5 after the procedure of pH change 7.5 → 10.6 → 7.5 was repeated 2 and 3 times, respectively (the incubation time at pH 10.6 was about 1 min). (B) The difference spectrum obtained by subtracting spectrum 1 from spectrum 4 (Fig. 2A). (C) M-Intermediate formation measured at 400 nm for D115N bR at pH 7.5 (curve 1). M-Formation measured at pH 7.5 (curve 2) and 5.5 (curve 3) after a 5-h incubation at pH 10.6. (D) The curve obtained by subtracting curve 2 from curve 1 (Fig. 2C) and its approximation by the exponential with a time constant of 249 μs. (E) Absorbance at 568 nm of D115N bR vs. M amplitude at 400 nm, pH 7.5. Points 1, 2 and 3 indicate relative values obtained when the procedure of pH changing 7.5 → 10.6 → 7.5 was repeated 1, 2 and 4 times, respectively.

strates the absorbance spectra measured at pH 7.5 (curve 1) and when the procedure of pH increase to 10.6 and decrease back to 7.5 was repeated two (curve 5) and three (curve 6) times. The transformation of the protein into the bR460 form was complete in 3–5 min (the time taken to adjust the pH). Curves 3 and 4 in Fig. 2 represent the spectra of D115N bR after a 5-h incubation at pH 10.6 and measured at pH 10.6 and 7.5, respectively. The same spectra were obtained when the procedure of pH change was repeated 4 times. This finding allows one to observe the correlation between the maximum of the absorbance spectra and the amplitude of the M-intermediate.

Fig. 2C shows M-intermediate formation at 400 nm, pH 7.5, before and after the 5-h incubation at pH 10.6. The amplitude decrease of 50% was observed after the formation of bR460. 90–95% of the 250-μs component disappeared, while the 60-μs component did not change significantly. The absorbance spectra of D115N bR and M-intermediate formation at 400 nm were measured at pH 7.5 during a pH increase to 10.6 and immediate lowering to 7.5. The decrease in the amplitude of the absorbance at 568 nm occurred concomitantly with the disappearance of the 250-μs component of the M-formation (Fig. 2E). We have concluded that the irreversible bR568 $\rightarrow$ bR460 transition features the bR molecules which form the M-intermediate with a time constant of 250 μs.

A comparative analysis of M-intermediate formation and decay of the D96N mutant at pH 7.0 and 4.6 was reported in [8]. The authors observed a mixture of L- and M-intermediates 3 ms after the flash at pH 4.6, and the L/M ratio was constant during the rest of the photocycle. If this phenomenon is of the same nature as the one described herein for D115N bR, it is interesting that the L-intermediate decay in the photocycle of the second form of bR occurs concomitantly with the M-decay of the first form at pH 4.5–5.5 for WT bR as well as for the D115N and D96N mutants. Assuming the existence of two forms of bR it follows that the D96N mutation decreases the decay rate of both the L- and M-intermediates in parallel photocycles and this may indicate a possible role for D96 in stabilizing the transition state of the protein.

The possibility that half of the D115N bR forms bR460 at pH 10.6 and the stability of this form at pH 7.5 allows one to demonstrate the heterogeneity of the bR pool at neutral pH. This is in line with our hypothesis previously postulated, on the grounds of spectroscopic differences between the two L-intermediates of the WT and D96N bR and on the basis of the two azide-independent phases of the L-decay of the D96N bR, that the ratio of two kinetically separate phases of M-intermediate formation, the 55–60 μs and 220–250 μs components, may be an indicator for the ratio of the two forms of the protein in solution. From analyzing the data obtained for different bR mutants we would like to note that the ratio between the two forms of bR varies for different mutants. The similarity of the two phases of the M-formation of D115N bR and WT bR (the same time constants, and pK's of 2.5 and 6.2 for the transition into the forms lacking the M-intermediate in the photocycles) indicates that WT bR comprises two bR forms at neutral pH as well as D115N bR. The form of the WT bR where M-intermediate formation occurs with a time constant of 220–250 μs exhibits a higher stability than the analogous form of the D115N bR.

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