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M-decay in the bacteriorhodopsin photocycle: effect of cooperativity and pH

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Abstract

The dependence of the bacteriorhodopsin (bR) photocycle on the intensity of the exciting flash was investigated in purple membranes. The dependence was most pronounced at slightly alkaline pH values. A comparison study of the kinetics of the photocycle and proton uptake at different intensities of the flash suggested that there exist two parallel photocycles in purple membranes at a high intensity of the flash. The photocycle of excited bR in a trimer with the two other bR molecules nonexcited is characterized by an almost irreversible M → N transition. Excitation of two or three bR in a trimer induces the N → M back reaction and accelerates the N → bR transition. Based on the qualitative similarity of the pH dependencies of the photocycles of solubilized bR and excited dimers and trimers we proposed that the interaction of nonexcited bR in trimers alters the photocycle of the excited monomer as compared to solubilized bR and the changes in the photocycles in excited dimers and trimers are the result of decoupling of this interaction.

Keywords: Bacteriorhodopsin; Photocycle; Proton transport; Cooperativity; Purple membrane; *Halobacterium halobium*

1. Introduction

It is well known that the rate of the M-intermediate decay in purple membrane depends on the intensity of the exciting flash [1–6]. Despite this fact, the problem is ignored in many works and photochemical conversions usually measured at high flash intensity are described as a linear sequential photocycle which includes both reversible and irreversible steps [7–13] or as a sum of independent photocycles lacking reversible steps [14]. Our experiments led us to propose that at least two independent photocycles may exist in a purple membrane simultaneously. The ratio between them depends on the excited flash intensity. One of them belongs to excited bacteriorhodopsin (bR) in a trimer in which the two other bR molecules are nonexcited. The other one is attributed to photochemical conversions of excited bR in a trimer in which two or three bR molecules are excited. The sequence of reactions M → N → bR is present in both photocycles. However, in comparison to one excited bR in the trimer excitation of two or three molecules in the trimer leads to the acceleration of the N → bR transition and to the appearance of the back N → M reaction.

2. Materials and methods

All experiments were carried out using freshly prepared purple membranes from *Halobacterium*

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halobium ET 1001. The purple membrane preparation, photocycle registration and purple membrane solubilization in 2% Triton X-100 were described elsewhere [3,15–17]. The pH responses associated with the bR photocycle were measured with pH indicators as *p*-nitrophenol [16] and pyranine [18]. All measurements were done at 20° C.

bR was excited with a neodymium Q-switched laser with a doublet light frequency (532 nm, $t_{1/2}$ = 15 ns, energy in green light 20 mJ) or IFK-120 photoflash with a GS-17 filter (> 500 nm, light impulse energy 20 mJ, $t_{1/2} = 150$ μs). The energy of the excitation flash was changed using neutral filters.

In experiments with D_2O the pD values of the deuterated samples were adjusted to a value of about 0.4 above the indicated pH values determined with a calibrated pH electrode [19].

The fit of the experimental curves to the sum of exponentials was obtained with the DISCRETE program [20].

3. Results and discussion

The dependence of the M-decay on the flash intensity was especially clear-cut at slightly alkaline pH values [3]. The transition from M to bR at a high pH included at least three kinetically distinct phases. The first two were clearly visible in the M-decay kinetics at 400 nm and were referred to as M-fast and M-slow [1–6], while the third slowest component was pronounced at 570 and 330 nm and was attributed to the N(P, R) → bR transition [17,21,22].

Increasing the flash intensity gave rise to the M-slow, which was practically absent at low flash intensity. The flash intensity had no effect on the rate constant of this component and affected just its amplitude contribution. Increasing the flash intensity led to a decrease in the contribution of the N → bR phase. The rate constant of this phase was also independent of the flash intensity. This component was not detected at 400 nm, but was pronounced at 330 nm, where the so-called β-band of the bR spectrum was localized, which is highly sensitive to the chromophore isomeric state [17,23]. The increase in the optical density at 330 nm was associated with K and L intermediates formation. We suppose that

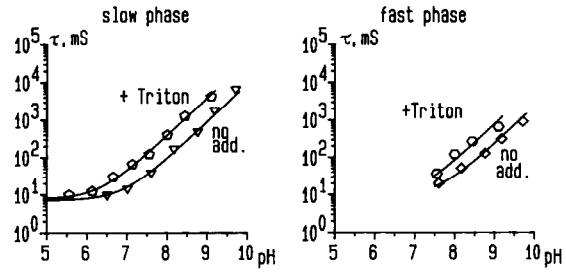

Fig. 1. The pH dependence of the two phases of photoresponse relaxation at 330 nm and the effect of 0.02% Triton X-100 on the pH dependence. Probe composition: 10 μM bR (purple membrane), 2 M NaCl, 20° C. The excitation laser flash intensity was 15 mJ/cm^2.

the signal decay at 330 nm reflects the *cis–trans* isomerization of the chromophore. At low flash intensity, only one slow component corresponding to the N → bR transition was detected in the relaxation of the signal at 330 nm. Increasing the flash intensity gave rise to the second component at 330 nm with the same time constant as for the M-slow. The contribution of this phase at 330 nm increased along with the increase in the contribution of the M-slow at 400 nm. All our observations on the flash intensity depending changes in the photocycle kinetics are in good agreement with the data of other works on this problem [1,4,5].

The M-slow and N-decay showed similar pH dependencies of their rate constants elucidated from the photoresponses at 330 nm at high flash intensity. At high pH both pH dependencies had slopes in logarithmic coordinates approaching to one (Fig. 1).

We failed to obtain reproducible pH responses associated with the bR photocycle at high pH using different pH indicators. So, we used another approach based on the effect of Triton X-100 on the bR photocycle. At a high ionic strength, a low concentration of Triton X-100 slightly accelerated the M-decay and decelerated both phases of the photoresponse relaxation at 330 nm due to shifting both pH dependencies to the low pH (Fig. 1). This allowed us to measure the pH responses at a neutral pH using *p*-nitrophenol and pyranine and to compare its kinetics to that of the bR photocycle. Identical results were obtained for both indicators.

During the time of experiment (1 h) Triton treatment does neither affect the sedimentation rate nor

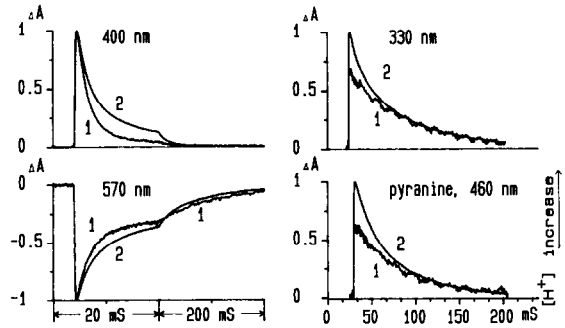

Fig. 2. Comparison of the photoresponses measured at low (1) and high (2) laser flash intensity (1 and 15 mJ/cm^2, respectively). Probe composition: 10 μM bR (purple membrane), 1 M NaCl, 0.02% Triton X-100 pH 7.2, 20° C. For measurements of the pH responses 100 μM pyranine was added.

the biphasic band shape of circular dichroism spectra in the visible region of purple membrane (data not shown). If this shape of the spectra is really due to the exciton coupling of bR molecules (for instance, see [24]), one can conclude that treatment by Triton at low concentration does not affect the arrangement of bR molecules in the purple membrane. A mechanism for the Triton-induced shift of the pH dependency to the low pH is not known. We suggest that incorporation of Triton molecules into the lipid fraction and/or substitution of some purple membrane lipids by detergent occurs. Therefore, one can speculate that this changes the surface charge density and lowers the surface pH.

The dependence of the photoresponse on the flash intensity at pH 7.2 in the presence of low concentrations of Triton X-100 was found to be similar to that observed at pH 8.5 in the absence of Triton (Fig. 2). Kinetic analysis revealed two components in the proton uptake kinetics and the same components for the photoresponse relaxation at 330 nm at high flash intensity. At low flash intensity only one and the same component was necessary for the fitting of proton uptake as well as for the photoresponse relaxation at 330 nm.

The strong correlation between the proton uptake and the relaxation of the '*cis*' peak at high flash intensity and low temperature was shown in our laboratory earlier [17]. Now we confirmed our data for a higher temperature and also found that the proton uptake at low flash intensity as well as the relaxation at 330 nm consist of one and the same component.

Fig. 3 demonstrates the dependence of the M-slow contribution on the change of the excited bR fraction in the purple membrane at pH 8.5. Both the laser flash and the photoflash were used for the bR excitation. Time duration was different for flashes used. A mixture of K, L and M intermediates appears in the sample during the excitation by the 150 μs photoflash. Therefore two photon processes should take place. Nevertheless, such processes do not affect the M → bR stages. We have compared signals obtained using a photoflash at low intensity where the amplitude of M-intermediate was the same as for the signal obtained using the laser flash of high intensity. The identical kinetics of the M-intermediate relaxation as well as of the relaxation at 330 and 570 nm indicates that the 150 μs time duration of the photoflash has no effect on the relaxation kinetics.

The use of the photoflash allowed us to reach 80% excitation (Fig. 3). This phenomenon might be explained as follows: during the time course of the excitation, bR ↔ K photoinduced equilibrium is established. Therefore even at high laser flash intensity the portion of excited bR, which photocycle via the M-intermediate, is limited by the value of the K → bR photoinduced back-reaction. During the longer

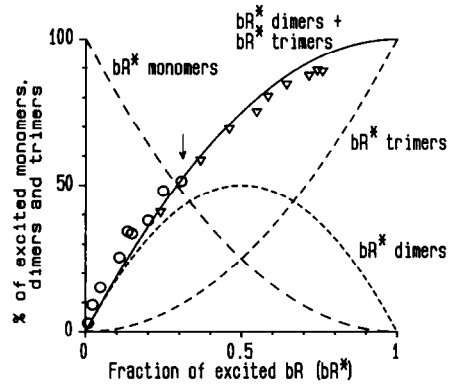

Fig. 3. The dependence of the contribution of excited monomers, dimers and trimers on the excited bR fraction in purple membrane. Experimental points (○, laser flash, ▽, photoflash) indicate the contribution of the pH dependent slow phase of the M-decay. Probe composition: 10 μM bR (purple membrane), 2 M NaCl, pH 8.5, 20° C. For details see text.

photoflash was used, the portion of bR which was formed from K was decreased due to the formation of M-intermediate and was further depleted during the flash . Thus we suggest that this is a reason why the saturation level of the photoflash is higher than that of the laser one.

The bR molecule entering into the photocycle including the formation of the M-intermediate is regarded as excited bR. Experimental points in Fig. 3, measured using a laser flash or photoflash indicate the contribution of the pH-dependent slow phase of the M-decay measured with different intensity of laser or photoflash. The arrow shows the point which was used for scaling of the experimental points on the basis of the contribution of the fast phase of the photoresponse relaxation at 330 nm. Fractions of excited bR were calculated on the basis of the amplitude of the M-intermediate (ϵ(M, 400 nm) = 30000 $M^{-1} \cdot cm^{-1}$ and ϵ(bR, 570 nm) = 63000 $M^{-1} \cdot cm^{-1}$). Theoretical curves demonstrate parts of the excited monomers (1 excited bR in a trimer), dimers (2 excited bR in a trimer) and trimers (3 excited bR in a trimer) of the excited bR pool. Equations used: $(1-x)^2$ for monomers, $2x^*(1-x)$ for dimers and x^2 for trimers, where x is the fraction of excited bR in the sample. Based on this experiment we came to the conclusion that the M-slow belongs to the photocycle of bR molecules in trimers, in which two or three bR molecules are excited simultaneously.

We suppose according to the above data that at high flash intensity two photocycles exist in the purple membrane. One of them is attributed to monomers. The other one to the photocycles of dimers and trimers. Fig. 4 demonstrates the difference between the M-decay kinetics for monomers and dimers (trimers). The curve for monomers was the experimental one measured at low flash intensity. The curve for dimers was the calculated one obtained by subtracting the monomer response from the experimental curve measured at high flash intensity. The coefficient for the monomer fraction was determined according to the contribution of the slowest component of the photoresponse relaxation at 330 nm. The curves for dimers and trimers were calculated using the experimental curves measured at high laser flash intensity and at high photoflash intensity. These curves were almost identical. Theoretical curves presented in Fig. 3 reveal different contribu-

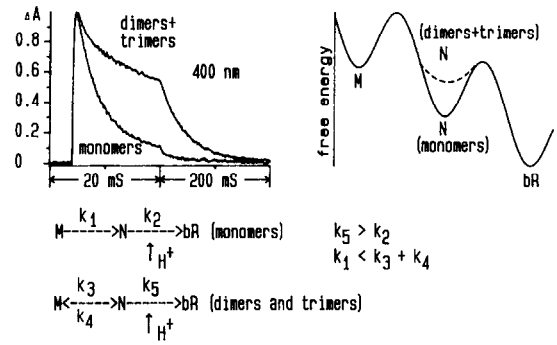

Fig. 4. Cooperativity effect in the bR photocycle in the purple membrane. For details see text.

tions of the excited dimers and trimers in the photoresponses at high and low flash intensity. So, one may conclude that excited dimers and excited trimers possess identical kinetic parameters of photochemical conversions. The M-fast was found to be present in the calculated photoresponse of dimers (trimers) with a 10–15% contribution. The M-fast for dimers and trimers is two times faster than that for monomers. We concluded that the M-fast is inherent in the photocycle of monomers as well as of dimers (trimers). For monomers it reflects an almost irreversible M → N transition. In the case of dimers and trimers it reflects the establishment of equilibrium M ↔ N. At high temperature the back reaction from N into M was induced in the photocycle of monomers. The N → bR transition for monomers is slower than (M + N) → bR for dimers. We suppose that the elevation of the energy level (destabilization of the N-intermediate) in the case of dimers (trimers) in comparison with the excited monomer may cause two effects: acceleration of the N → bR transition and N → M back-reaction. Both these effects were revealed in our experiments. We reinvestigated the difference spectrum of the M-slow and came to the conclusion that in spite of its similarity to the 'M minus bR' difference spectrum a small difference really exists. This difference can be due to the presence of 10–15% of the 'N minus bR' difference spectrum in the difference spectrum of the M-slow.

It should be stressed that the M ↔ N equilibrium in dimers is more shifted towards M at high temperature than at low. In our opinion, that is why Tokaji and Dancshazy [4] did not observe the N-intermediate, corresponding to the M-slow using the

method involving the photoreaction of the N-intermediate. We suppose that the N-intermediate in the photocycle of dimers is identical to the N^{-1} intermediate denoted by Zimanyi et al. [11] while N in the photocycle of monomers is identical to N^0. According to scheme suggested by Zimanyi et al., N^{-1} transforms into N^0 after the protonation, and the transition of N^0 into bR is not accompanied by the proton uptake and associated with the *cis–trans* isomerization. According to our experiments, analysis of small pH responses at high pH is ambiguous. We suggest that pH responses measured at neutral pH in the presence of Triton provide more adequate information. We can not exclude that the N to bR transition really occurs in two steps. Moreover, we observed [25] that the M-decay of the D96N mutant in the presence of azide was accompanied by the proton uptake from the bulk phase measured with *p*-nitrophenol. At the same time the N-decay proceeded much more slower and did not depend on pH. This was direct evidence of the existence of two steps: restoration of the initial protonation state of the protein is followed by the pH-independent N → bR transition associated with the *cis–trans* isomerization of the chromophore group. Elucidation of the isomerization process is due to its retardation in the mutant protein (half-time is about 40 ms). Nevertheless, in our opinion the proton uptake in the photocycle of the wild-type protein is a rate-limiting step and the isomerization processes cannot be separated kinetically at a high pH.

An interesting phenomenon was observed during our experiment on the sensitivity of the bR photocycle to H_2O for D_2O substitution (Fig. 5). D_2O induces a 5-fold deceleration of the M formation and a 2-fold deceleration of the M to N transition. At the same time, D_2O accelerates the N → bR transition at high pD. Both phases of the photoresponse relaxation at 330 nm are accelerated in D_2O. To our mind, this phenomenon can be understood if one assumes that protonation of the M-intermediate occurs with the participation of some group. In this case the rate constant of the N → bR transition (k_1) will be proportional to the concentration of the protonated form of this group ($k_1 = k \cdot [AH]$). It is known that pK of acidic groups is increased in D_2O [19]. This must result in a increase in the concentration of the protonated form of an acceptor and

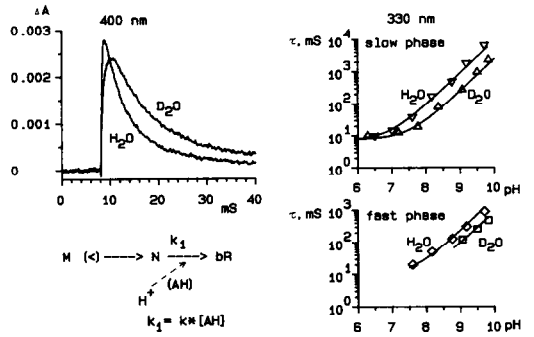

Fig. 5. Effect of D_2O on the photoresponses at 400 nm measured at low laser flash intensity (1 mJ/cm^2) and on the two phases of relaxation of the photoresponses at 330 nm measured at high laser flash intensity (15 mJ/cm^2). Probe composition: 10 μM bR (purple membrane), 2 M NaCl, 20° C.

therefore in the increase in the rate of protonation. Noteworthy is the tendency for saturation of the pH dependence of the rate of the N → bR transition at low pH. Really, experimental points for the slow phase in H_2O can be approximated well by a theoretical curve reflecting the titration of a group with a pK of about 6.9. The simplest assumption is that this group is Asp-96 itself. Then, k is the rate constant of the *cis–trans* isomerization and the measurable pK (6.9) is the pK of Asp-96 in the excited state of bR. However, at present we cannot exclude a more complicated situation that this group is another proton–acceptor group which serves as an intermediate group during the protonation of the N intermediate and transfers a proton from the bulk phase to Asp-96. In any case, our data on the D_2O effects point to the functional similarity between the M-slow and the N → bR transition and to the difference between the M-slow and M-fast phases.

The photoresponse of bR solubilized in 2% Triton X-100 does not depend on the flash intensity [3]. Fig. 6 shows the pH dependence of the M-slow contribution for bR solubilized in 2% Triton. It must be remembered that the contribution of this phase depends on the ratio of the time constants of forward and back reactions in the M ↔ N equilibrium [3]. This pH dependence reveals two transitions. The rate of this phase shows the tendency to be saturated in the pH region of the low pH transition. We suppose that the protonation state of the intermediate proton–acceptor group may also determine the low

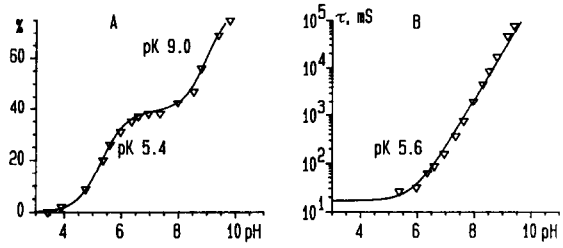

Fig. 6. The pH dependence of the contribution (A) and the rate (B) of the slow phase of the M-decay in the photocycle of solubilized bR. Probe composition: 10 μM bR solubilized in 2% Triton X-100, 100 mM NaCl, 2% Triton X-100, 20° C.

pH transition (according to the above mentioned simplest assumption, this pH transition may reflect the pK of Asp-96 in the excited state of bR).

The pH dependence of the M-slow contribution of the purple membrane at high flash intensity shows a qualitatively similar pattern (Fig. 7). Its main distinctions from solubilized bR are that the pKs of both transitions are localized at higher pH and that the transitions take place in a narrower pH region. The former difference may be partially associated with the extraction of acidic lipids during solubilization. The narrower interval of pH transitions may point to their cooperative character in the purple membrane. The pH dependence at low flash intensity almost lacks the low pH transition (not shown). The pH dependence of the M-photoresponse in the purple membrane is more similar to that of solubilized bR at high flash intensity than at low flash intensity. This phenomenon raises the question about the mechanism of the cooperativity effect. There are two possibilities. The first one is that the interaction between excited bR molecules causes changes in the bR photocycle. The second one is that the changes in the photocycle in dimers and trimers are the result of decoupling. The interaction of nonexcited molecules alters the photocycle of the excited monomer as compared to solubilized bR. On the one hand, it gives an insight into why there are no detectable differences between excited dimers and trimers. On the other hand, it allows one to explain the existence of the M-slow in the photoresponse at a very low flash intensity in terms of distortions in the crystalline array in purple membranes. The contribution of the M-slow at low flash intensity usually does not exceed several percent but depends to some extent on preparations and increases at high temperature.

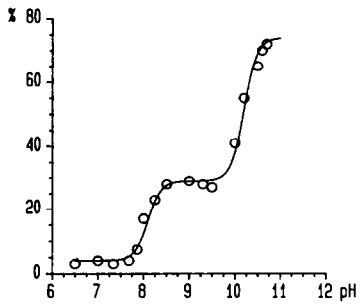

Fig. 7. The pH dependence of the contribution of the pH-dependent components of the M-decay in the bR photocycle measured at high (15 mJ/cm^2) laser flash intensity. Probe composition: 10 μM bR (purple membrane), 2 M NaCl, 20° C.

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