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Kinetics of the N intermediate and the two pathways of recovery of the ground-state of bacteriorhodopsin

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Absorption kinetic measurements at alkaline pH, in which bacteriorhodopsin (BR) is pre-excited by another flash, indicate that a part of the recovery of the BR ground-state is faster than the decay of the N intermediate of the photocycle. This fact proves the existence of a parallel pathway in the late part of the BR photocycle (the decay of M_s into BR), which does not include the N intermediate. We demonstrate that the decay of the M_f intermediate does not lead to any direct recovery of the BR ground-state, and that excitation of N does not form an M-like intermediate. M_f decays directly into the N intermediate, and photoexcitation of N leads to the formation of a red-shifted form, O*. The kinetics of this red-shifted intermediate are also presented.

Bacteriorhodopsin; Decay of the N intermediate; Ground-state recovery; Photocycle; Yield of the M intermediate

1. INTRODUCTION

Bacteriorhodopsin (BR) is a light-driven proton pump (for a recent review see e.g. [1]). A comprehensive model of the BR photocycle, which would be able to explain the experimental results obtained under a wide range of experimental conditions, and would be widely accepted, has not yet been established. Some of the kinetic features of the BR photocycle in the millisecond time range (biphasic decay of the M intermediate, location of the recently rediscovered N intermediate, and the unusual behavior of the O intermediate under different circumstances) have been explained by introducing branchings [2,3] and back-reactions [4,5] in the photocycle models, or by supposing more than one thermally equilibrating BR ground-state with independent photocycles [6,7]. Recent studies on the absorption changes accompanying the BR photocycle indicated the complexity of the kinetics and the pending nature of these possible explanations [8].

The most basic information which could answer the possible pathways of the photocycle would be the amount of each intermediate at each time during the cycle. These time dependencies of the concentrations of the individual intermediates were studied directly only in connection with the photocycle model suggested in [5].

Recently an O-like intermediate induced by excitation of the N intermediate has been reported [5,9]. As we will show below by this photoreaction, in absorption kinetic measurements using pre-excitation [10], the amount of N intermediate formed during the photocycle can be measured directly.

By the double-pulse method the recovery of the ground-state of BR can be determined, too [11–13]. These experiments indicated that, on the one hand, the kinetics of recovery of the BR ground-state and the millisecond part of the absorption changes at 335 nm coincide, and on the other hand, that at alkaline pH these signals can be described by two exponential components, of which only the faster one participates in the kinetics of the M intermediate (detected at 400 nm). Besides this common component, an additional, faster component of the M decay was also found.

The results and the comparison of these two double-pulse experiments are presented in this paper.

2. MATERIALS AND METHODS

Preparation of the sample and the absorption kinetic measuring system were essentially the same as described in [10].

The pre-exciting flash was provided by a flash lamp pumped dye laser (Carl Zeiss, Germany; 1 μs half duration, rhodamine 6G dye, λ=590 nm). For the second excitation a nitrogen laser (Jate, Hungary; 10 ns half duration) pumped dye laser (rhodamine 6G) was used. The data were collected by a transient recorder with a logarithmic time base, described in [14].

The BR sample was incorporated in a 1.5 mm thick slice of 10% polyacrylamide gel, with an absorbance of 0.25 at 570 nm, using 30 mM universal buffer (citric acid, monopotassium sulfate, borate, diethyl-barbiturate) and 1 M NaCl.

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Abbreviations: BR, bacteriorhodopsin; M_f and M_s, rapidly and slowly decaying M intermediates; O*, red-shifted photoproduct of the N intermediate.

3. RESULTS AND DISCUSSION

3.1. *Kinetics of the N intermediate and of its photoproduct*

The red-shifted photoproduct (O*) of the N intermediate, the difference spectrum of which has been published in [5], is well detected at 670 nm in absorption kinetic experiments using pre-excitation, as can be seen in Fig. 1a. At alkaline pH, without pre-excitation, only small absorption changes are induced by single flashes (in the 0.1–1,000 ms time range), as under these conditions the formation of the O intermediate is insignificant. Using pre-excitation with an appropriate delay time between the two flashes, a strong positive peak appears (Fig. 1a), with a maximum at ca. 200 μs after

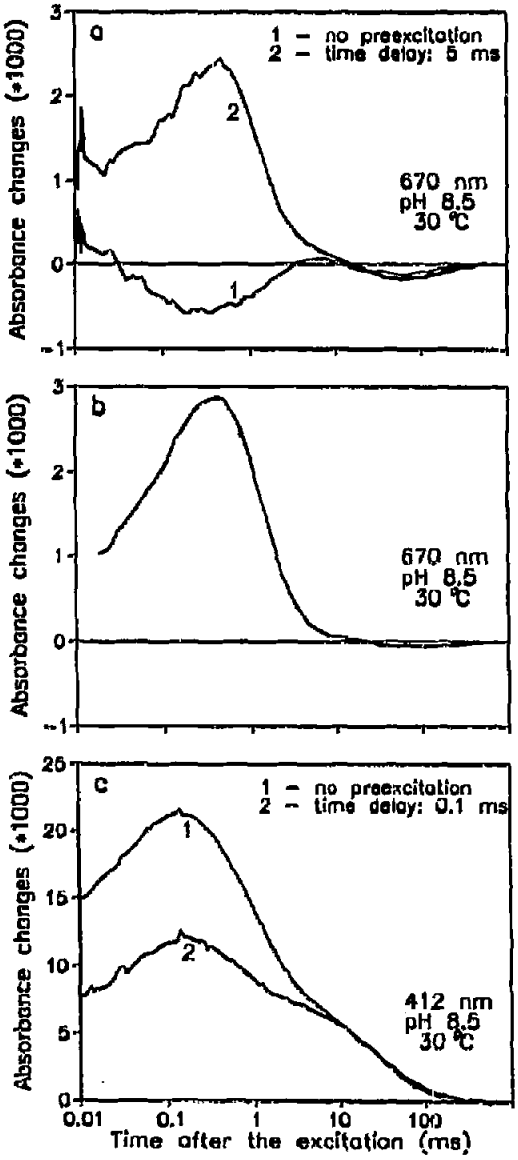

Fig. 1. Influence of pre-excitation on the absorption changes at (a) 670 nm and (c) 412 nm. (b) The kinetics of the photoproduct of the N intermediate.

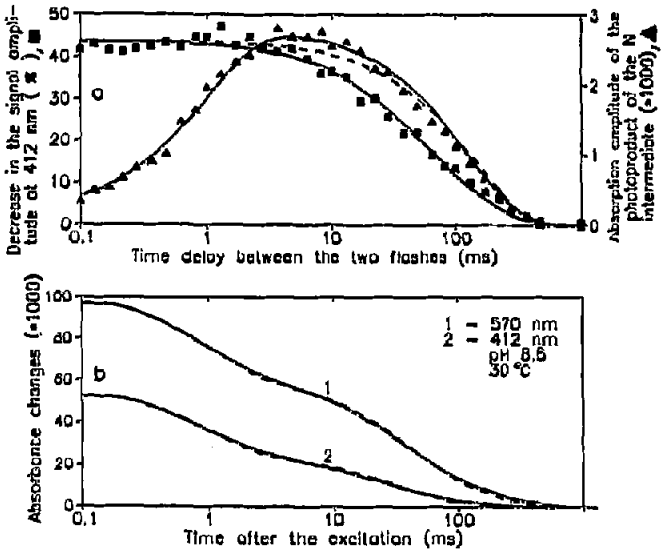

Fig. 2. (a) Recovery of the BR ground-state, indicated by the decrease in M-forming ability (■), and the kinetics of the N intermediate (▲), determined by the yield of its red-shifted photoproduct. The solid lines are the best fits with the 1, 30, and 120 ms components (see text). The amplitudes are listed in Table I. The dashed line indicates the expected recovery of the BR ground-state, if it takes place only by the decay of N. (b) The kinetics of the absorption changes induced by pre-excitation at 412 and 570 nm (solid lines) fitted with the 1, 30, and 120 ms components (dashed lines). The 570 nm signal is inverted.

the second excitation, indicating the suggested N→O* photoreaction. (Fig. 1b and c will be discussed later.)

The yield of O* (i.e. the amplitude of the positive peak after subtracting the absorption changes induced by the pre-excitation itself, and those (small) ones, which originate in the excitation of the remaining part of the BR ground-state by the second flash at 670 nm) strongly depends on the delay time between the two flashes, as it can be seen in Fig. 2a. (indicated by triangles). By exponential fitting, 1 and 110 ms time constants were found (with equal amplitudes within the experimental error) for its rise and decay vs. the delay time. Since the absorption changes detected at 670 nm after the second flash originate in the N→O* photoreaction, these time constants describe the formation and the decay of the N intermediate (which was generated by the pre-excitation, and was excited again by the second flash).

The decaying part of the corresponding absorption changes detected at 570 nm (the signal reflecting the recovery of the absorption at the peak of the BR main absorption band) induced by the pre-exciting flash alone (shown in Fig. 2b) was fitted with 3 exponentials. In this way 1, 25, and 130 ms lifetimes were found for the decays of the M_f, M_s, and N intermediates, respectively (as these components are often assigned, e.g. in [6,15]).

The good agreement between the lifetimes of M_f and N in the two different experiments confirms that O* is

Table I

The amplitudes and relative weights (in parentheses) of the different components of the second part of the BR photocycle in the different kinetic signals shown in Fig. 2. (M_f 1 ms, M_s 30 ms, N 120 ms)

Signal	M_f	M_s	N
412 nm	33.0 (57.4%)	21.5 (37.4%)	3.0 (5.2%)
570 nm	−41.8 (40.3%)	−36.5 (35.2%)	−25.4 (24.5%)
BR recovery	–	18.0 (41.2%)	25.7 (58.8%)
N kinetics*	−2.66	–	2.81

The amplitudes are given in the same units as the corresponding signals in Fig. 2.
Given in arbitrary units; measured by the N→O photoreaction.

the photoproduct of the N intermediate (as was suggested in [5] and [9]) and that N is formed only by the decay of M_f.

The kinetics of O* determined from the data presented in Fig. 1a are shown in Fig. 1b. In our experimental conditions the formation and the decay of this photoproduct can be described by 140 μs and 1 ms time constants, respectively. These values are in a good agreement with those (100 μs and 1.7 ms at 22°C) reported in [9] for this O-like intermediate (denoted there as Q).

3.2. *Kinetics of recovery of the Br ground-state*

By pre-excitation of the sample before the beginning of the absorption kinetic measurement, the amplitude of the absorption changes naturally decreases but the relative weight of M_s increases [10] as can be seen in Fig. 1c (5–50 ms region).

The decrease in the maximum amplitude of the absorption changes in the signal induced by the second excitation vs. the time interval between the two excitations detected at 412 nm is shown in Fig. 2a (by squares). As it has reported several times [11–13] this decrease in the M-forming ability of the sample (during the photocycle, initiated by the pre-excitation) is equal to the cycling fraction, and its disappearance describes the recovery of the Br ground-state. The following facts are indicated by this curve.

(i) No recovery of the ground-state can be found by the decay of M_f (τ = 1 ms). (On the other hand the kinetics of N in Fig. 2a indicate that M_f decays into N).

(ii) Excitation of N does not form an M-like intermediate, otherwise any N→M-like photoreaction would yield an increase in the M-forming ability, which should be seen by the decay of M_f (i.e. by the accumulation of N).

(iii) Fitting of a single exponential to the recovery kinetics of the BR ground-state gives a poor approximation with a 62 ms time constant, while the sum of two exponentials describes the process well (30 and 130 ms lifetimes), in accordance with [13]. This indicates that a part of the recovery of the BR ground-state is faster than the decay of the N intermediate (τ = 130 ms). This is a strong argument for a parallel pathway in the BR photocycle leading to the ground-state which does not include the N intermediate. The fact that the only source of recovery of the BR ground-state can not be the decay of N (by a first order reaction) described by the $d[BR] = k \cdot N \cdot dt$ differential equation (where k is the rate constant of the N→BR decay) is demonstrated also by the expected BR recovery kinetics (dashed line in Fig. 2a) calculated from the integral of the measured N(t) function (Fig. 2a, triangles): as can be seen, a considerably slower ground-state recovery would be expected than was found.

A great number of schemes have been suggested recently to describe the second part of the BR photocycle at alkaline pH, representatives of which are shown in Fig. 3. From these schemes the O intermediate is now omitted, as under our measuring conditions its presence practically cannot be detected, and so it may not influence the kinetics of the other intermediates to any extent.

Scheme a in Fig. 3 (suggested e.g. in [6,7]) is in contradiction with finding (i), as the decay of M_f should lead

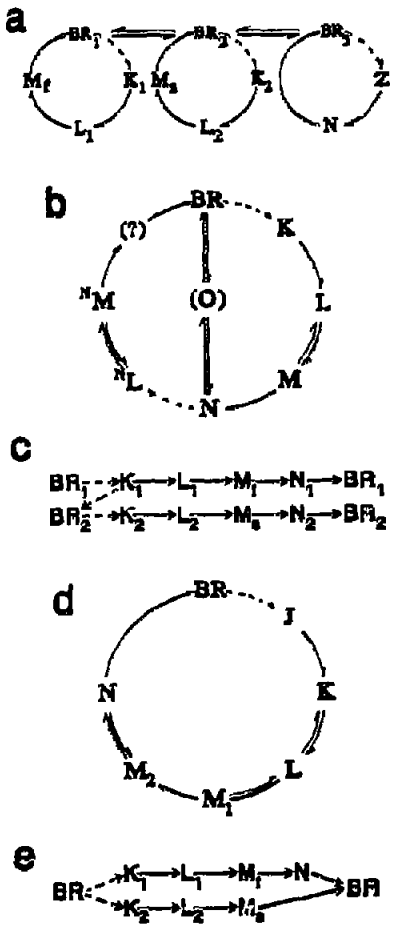

Fig. 3. Representatives of the schemes suggested recently (taken from (a) [6], (b) [15], (c) [16], (d) [5] and (e) [3]) for the second part of the BR photocycle.

to some recovery of the ground-state. Scheme b (introduced in [15]) suggests an M-like photoproduct of the N intermediate, which would be in contrast with finding (ii). Schemes c and d (suggested e.g. in [16] and [4,5,8], respectively) disagree with finding (iii), as the only source of the recovery of the BR ground-state should be the decay of the N intermediate. (Note that scheme c could be suitable if N_2 is either extremely short lived, or is active in forming M and inactive in forming O* by excitation.)

Scheme e is in accordance with our results. The participation of M_s in the M decay is considerable (Fig. 2b, 412 nm), but this process is not reflected in the $M_f \rightarrow N \rightarrow BR$ pathway (Fig. 2a, triangles), thus it must be included in the other, parallel regeneration pathway of the ground-state. On the other hand, the decay of M_s really participates in the recovery of the BR ground-state (Fig. 2a, squares).

The agreement of scheme e with the experimental data was further analyzed. The lifetimes of M_f, M_s, and N were taken as 1, 30, 120 ms (these are average values of the slightly different lifetimes determined from the different signals) and their amplitudes were determined in the different kinetic signals. The best fits with these lifetime values and the corresponding amplitudes of the components are shown in Fig. 2a and b and in Table I, respectively.

The relative weight of M_s in the absorption changes at 412 and 570 nm and in the BR recovery, according to the model, should be equal to the percentage participation of the M_s-containing pathway in the photocycle. At 412 nm, 570 nm (Fig. 2b), and in BR recovery it is 37.4, 35.1, and 41.3%, respectively. These data confirm the validity of scheme e and indicate that $37.9 \pm 3.1\%$ of the initially cycling molecules recovered through M_s (at the actinic light density provided by the pre-excitation).

In conclusion we found only one way to describe the kinetics of the N intermediate compared to the recovery of the BR ground-state, indicating that in the photocycle of BR, the M_f intermediate is followed by N, while the M_s intermediate, without a subsequent N, decays into the ground-state, process that was suggested from different considerations e.g. in [3].

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