Actinic light density dependence of the O intermediate of the photocycle of bacteriorhodopsin

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Abstract The O intermediate of the photocycle of bacteriorhodopsin (BR) was studied by absorption kinetic measurements at different actinic light densities. With increasing exciting flash intensity, the relative yield of O slightly increases, while that of M_f strongly decreases at the expense of M_s. Kinetic calculations and the optical anisotropy of O show that O can be formed only from M_f although M_f and O have different light intensity dependences. In order to resolve the apparent contradiction, a phenomenologically new cooperative regulatory mechanism seems to be necessary.

Key words: Halobacterium halobium; Light intensity dependence; M intermediate decay; Parallel pathways; Photocycle model; Photocooperativity

1. Introduction

Several recent studies have been devoted to elucidating the features of the bacteriorhodopsin's photocycle (for recent reviews see [1,2]). The millisecond time domain of the photocycle has provided at least two surprises during the recent years. One was the discovery of the N intermediate in 1986–88 [3-5], the other being the demonstration of the light intensity dependence of the photocycle kinetics [6,7]. This latter phenomenon has been extensively studied under conditions of high pH where, in the absence of accumulation of the O intermediate, it manifests as changes in the relative weights of the two components of the M decay, and in the relative amount of the N intermediate (see e.g. [8]).

The origin of the biphasic decay of the M intermediate at high exciting light intensity has been described based on the frame of photocooperativity that exists among the BR molecules belonging to the same trimeric unit [9], and the light intensity dependent millisecond part of the BR photocycle at high pH with a model consisting of two cooperativity-regulated ($M_f \rightarrow N \rightarrow BR$ and $M_s \rightarrow BR$ [8], or $M \rightarrow N \rightarrow BR$ and $M \leftrightarrow N \rightarrow BR$ [10]) parallel pathways. Other attempts have been made to describe the actinic light density dependence of the photocycle kinetics by cooperativity in some recent works [10-12].

At about neutral pH or below, the O intermediate also accumulates in the photocycle. The position of the O intermediate in the photocycle is under debate, and its light intensity dependence is unknown. In structural respects, it has been suggested that the appearance of the O intermediate is due to the fact that an amino acid of BR, Asp-85, is transiently in a protonated state (see e.g. [1]).

A recent point of view regarding the correlation between the O and M intermediates [13] favours the idea that M_f decays directly to O, whereas the decay of M_s restores the ground state of the BR molecules. However, previous studies suggested a rapid equilibrium between M_s and O [14] or O and N [15].

In another recent study, two different pH-regulated pathways were suggested for the accumulation of O and N, respectively [1]. Although according to that model both pathways contain more than one N and O state, at high pH N accumulates mainly whereas O can be seen at low pH.

In the present study the fact that the actinic light density regulates the relative yields of the components will be used as a tool for investigating the features of the O intermediate. The important finding is that O is the subsequent intermediate of M_f, with an unexpected light intensity dependence. Interestingly, the problem seems to be solvable only by the assumption of an additional cooperative regulation.

2. Materials and methods

For performing absorption kinetic measurements, the preparation of the sample and the technical features of the measuring system were the same as described in detail elsewhere [9,16]. For excitation an excimer laser pumped dye laser containing coumarin 307 ($\lambda_e = 505$ nm) was used [6].

Unless otherwise indicated, measurements were carried out at pH 7.0, 1 M NaCl salt concentration and 30°C. For improving the signal-to-noise ratio, the curves are the average of at least 100 repetitions. The computer program used for kinetic simulations was written in Turbo Pascal 6.0, by one of the present authors (T.L.).

The dichroic ratios were calculated as the ratio of the absorption changes measured for parallel and perpendicular polarization of the measuring beam to the actinic beam.

3. Results and discussion

The M intermediate decay is biphasic (Fig. 1a) at any actinic light density used for the excitation of BR (the relative intensities used were 0.01 and 1 for the weak and strong laser flash, respectively). The results of the data evaluation with exponential components indicate that the lifetimes of the M decay components (M_f and M_s) are independent of the actinic light intensity (M_f, 1.0 ms; M_s, 6.1 ms), while the relative weight of M_s increases with increasing actinic light intensity (Fig. 2a, triangles). These are in accordance with our previous results [9,16], and the observed phenomenon has been tackled in the frame of the cooperative interactions among neighbouring BR molecules.

Fig. 1b shows the absorption changes at 680 nm at the same actinic light densities as in the case of Fig. 1a, normalised with

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Abbreviations: BR, bacteriorhodopsin; M_f, M_s, N and O, intermediates of the photocycle.

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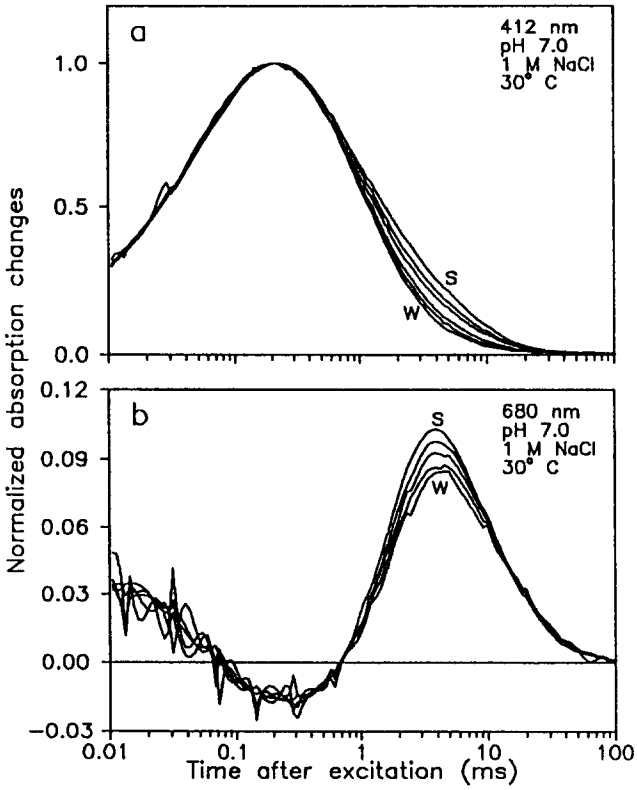

Fig. 1. The transient absorption changes characteristic for M, normalized to their maximum value (a) and for O, normalized with the same factor used for the normalization of the corresponding 412 nm kinetics (b), at different exciting light intensities; 30°C. Unlike the relative yields of M_f, M_s and O, the lifetimes corresponding to the decay of M_f, M_s and O are practically independent of the flash intensity (M_f, 1.0 ms; M_s, 6.1 ms; O, 12.6 ms). The relative flash intensities were 0.01 (W, weakest) and 1 (S, strongest), respectively.

the same factors as used for the corresponding 412 nm data. In this representation, the amplitude of the small negative peak in the kinetics at 680 nm is constant, thus without normalization the amplitude of this small negative peak is proportional to the amount of M. The positive peak reflects the amount of O accumulated during the photocycle. Note that when strong flashes were employed for the excitation of BR, the relative yield of O is greater compared to the case of weak flashes.

In accordance with our previous studies [9,16], the relative amount of M_s increases proportionally to the M peak amplitude (and with the fraction cycling) as shown in Fig. 2a (triangles). Similarly, the increase observed in the relative yield of O seems to be proportional to the maximum amount of M formed during the photocycle, however, this change is notably smaller than the increase in the relative weight of M_s (Fig. 2a, circles).

The models for the description of the actinic light density dependence of the photocycle kinetics contain at least two parallel pathways, one with a rapidly decaying (M_f), and another with a slowly decaying (M_s) M form [8,17]. The simplest model able to predict an increase in the amount of O with increasing relative yield of M_s would entail both M_f and M_s generating O, but with different efficiencies [13].

According to this idea, the peak amount of O would be proportional to the weighted sum of the yields of M_f and M_s:

$$[O]_p = a \cdot (\alpha \cdot [M_f]_i \cdot 0.810 + [M_s]_i \cdot 0.507) \quad (1)$$

where α represents the O-forming efficiency of M_f compared to M_s, and a is a proportionality factor. $[Mf]_i$ and $[Ms]_i$ refer to the proportion of the photocycling BR molecules which form the M_f and M_s intermediate, respectively. The coefficients 0.810 and 0.507 give the maximum (peak) fractions of O for the case where unit amounts of the molecules pass through M_f and M_s, respectively (these factors were deter

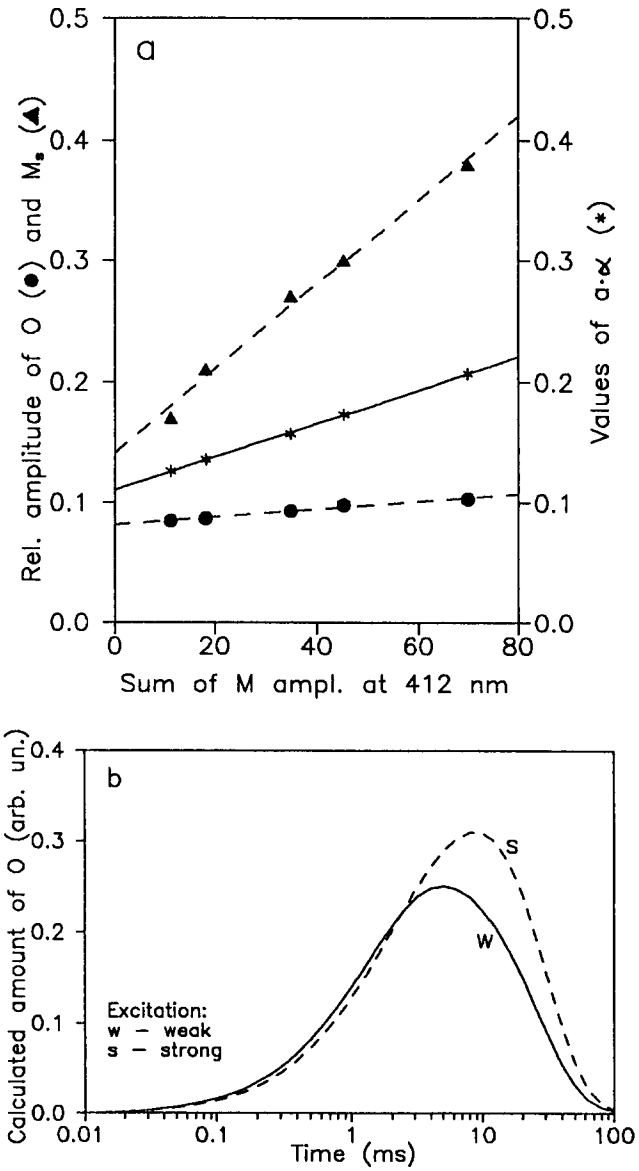

Fig. 2. (a) Dependence of the relative yields of M_s (▲, calculated as $[M_s]_i/([M_f]_i+[M_s]_i)$) and O (●, calculated as $[O]_p/([M_f]_i+[M_s]_i)$) upon the peak amount of M formed ($[M_f]_i+[M_s]_i$). The asterisks represent the values of $a\cdot\alpha$ (see text). (b) The simulated kinetics of the O intermediate for the weakest (W) and strongest (S) exciting flash intensities, as predicted for the case where both M_f and M_s form it with different efficiencies (see text). The lifetimes used in simulation those resulting from the original data fitting (M_f, 1.0 ms; M_s, 6.1 ms; O, 12.6 ms). The relative weights of M_f and M_s were obtained from the data shown in Fig. 1a.

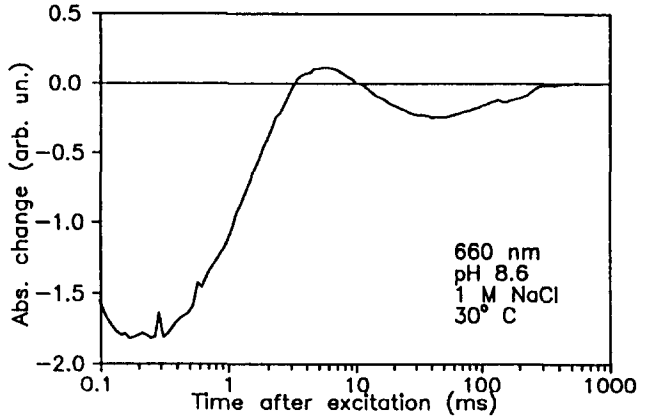

Fig. 3. Absorption changes measured at 660 nm, pH 8.6 and 30°C which suggest that when O almost completely decayed, there is still a significant amount of the BR molecules present in the N state (the negative peak in the late millisecond part). The fraction cycling was 16%.

mined in a simple simulation using the observed time constants (M_f, 1.0 ms; M_s, 6.1 ms; O, 12.6 ms).

Simple calculations with the data shown in Fig. 2a lead to the result that the dependence of the peak amplitude of O could be described satisfactorily by α=0.30 (i.e. the efficiency of formation of O by an M_f molecule would be 30% of that of an M_s molecule). However, the expected O kinetics for the weakest and strongest excitation would conflict with the data: Fig. 2b shows that at higher exciting flash intensities, the time when the maximum amount of O is formed should shift to higher values compared to the case of low exciting flash intensities. This expected shift is considerably larger than the experimental uncertainties and so the simulated kinetic feature of the O intermediate is in contradiction to the measured kinetics (Fig. 1b). As a consequence, the starting assumption that M_s may also decay to O seems to fail, in spite of the fact that the increase in the amplitude of O with increasing fraction cycling would allow that mechanism.

The only exceptions to this kinetic problem are those models in which the O intermediate would be formed in the M_s-mediated pathway with a very similar time constant to the lifetime of M_f. Such a scheme would be:

$$\begin{array}{l} \qquad O_1 \\ \qquad \updownarrow \\ M_f(\leftarrow) \rightarrow N_1 \rightarrow BR \\ M_s \longleftrightarrow N_2 \rightarrow BR \\ \qquad \updownarrow \\ \qquad O_2 \end{array}$$

if the back-reaction from N_1 to M_f is practically negligible (because the N decay is invisible at 412 nm), the equilibrium reactions between the O and N intermediates are relatively fast, and the amount of N_2 is always small. Note that this scheme without O has been suggested recently by Komrakov and Kaulen [10].

These types of model, although not showing the above-mentioned kinetic problem, appear to be contradicted by one fact. This is as follows:

The maximum absorbance change at 680 nm due to the accumulation of the O intermediate is about 10% of the maximum absorbance change due to M at 412 nm. On the basis of the absorption spectra of the intermediates [15] one can estimate the same amount of O from the photocycling molecules (under conditions similar to ours Váró and Lanyi measured a higher, approx. 15% O peak concentration [18]) As we now suppose that practically no difference exists in the kinetics of the O intermediates formed in the different pathways, we can use Eq. 1 without the coefficients 0.810 and 0.507. The result obtained from the calculations is that $\alpha = 0.43$, and if we take 10% as the mean value for the peak amount of O, then the same equation leads to the result that, when the amount of O is maximal, 17% of the molecules are in (and later decay as) O instead of M_s. In contrast, the light intensity dependence of the relative weight of M_s is independent of pH between 7 and 10 [16], while the amount of O increases with decreasing pH, therefore, the data do not reflect the decrease expected if a considerable amount of O were to be formed at the expense of M_s.

Note that the same problem would appear if one were to suppose that O is in direct equilibrium with M_s.

The result of the kinetic measurements carried out at 660 nm, pH 8.6 and 30°C (the case where M, N and O appear well separated in time due to the higher pH, and the extent of the absorbance changes due to O and N are similar to each other) is shown in Fig. 3. It is clearly evident that an appreciable amount of BR molecules are still in the N state (Fig. 3; the negative peak in the late millisecond part) while O (its appearance being indicated by the positive band in Fig. 3) at that time has almost completely decayed. This observation contradicts the possibility of an equilibrium between N and O. Additional evidence is that it has previously been shown that the amount of N decreases [16] whereas the amount of O increases with increasing actinic light density (Fig. 2a, triangles). This is in accordance with the suggestion by Lanyi in his recent review [1] that the traceable positions of N and O should be in different parallel pathways, although the light intensity dependence of the O intermediate is not expected from that model.

Since the data do not support the hypothesis that M_s could form O, and O cannot be in an actinic light density independent equilibrium with any of the intermediates, we had to

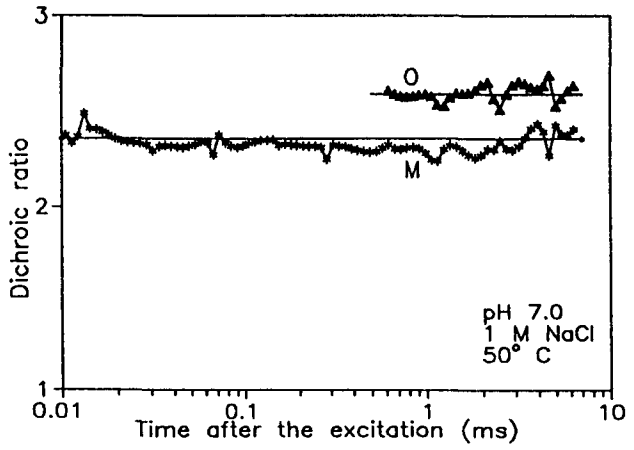

Fig. 4. The dichroic ratios at 412 and 690 nm that are characteristic for the M and O intermediates, respectively.

consider a model in which only M_f would decay into O. A kinetic reason (i.e. that the time constants for the accumulation of O and the decay of M_f agree well) also exists for this, as reported in a preceding paper [13] and confirmed in the present measurements.

We found further evidence for the contention that only M_f forms O which is based on the optical anisotropy of the intermediates. In a previous study, we have shown that the dichroic ratio for M_f is greater than for the whole M population in the microsecond time scale [19]. Fig. 4 shows that the dichroic ratio of O is also higher (with a value of 2.59–2.36 = 0.23). (In this measurement the wavelength was changed to 690 nm and the amount of O was multiplied by increasing the temperature to 50°C in order to have absorption changes practically completely from O, and a sufficiently good signal-to-noise ratio.) As in the present case the change in the dichroic ratio is not very pronounced at 412 nm due to the fact that when O can be seen the decays of M_f and M_s overlap, we have to estimate the dichroic ratio of M_f from the high pH case illustrated in Fig. 2a of [19]: In that experiment at 0.1 (0.05) flash intensity, the dichroic ratio of M was 2.25 (2.57) in the microsecond time scale, and after the decay of M_f the dichroic ratio (i.e. of M_s) was 1.80 (2.07). Simple calculation with these values (taking into account that the relative weight of M_f from the whole M population was 65 (70)%) leads to the result that the dichroic ratio of M_f is higher by 0.24 (0.22) than that of the whole M population. This value is exactly the same as observed for O in Fig. 4.

As several arguments exist for the view that only M_f forms O (despite their opposite actinic light density dependences), an additional cooperative event seems to be necessary in the millisecond part of the BR photocycle. According to this, the yield of O depends not only on the pH and temperature (see e.g. [1]), but also on the fraction cycling.

The dependence can be described as:

$$[O]_p = a \cdot \alpha \cdot [M_f]_i \cdot 0.810 \qquad (2)$$

where all of the designations are the same as in Eq. 1, but α refers to the proportion of M_f decaying through O, and this depends on the fraction cycling. The asterisks in Fig. 2a show the dependence of $a \cdot \alpha$ on the amplitude of M and this dependence seems to be linear within the experimental uncertainties. Taking into account that according to previous studies [8,9] the relative weight of M_s (triangles in Fig. 2a) has a slope of 0.5 vs. the fraction cycling (FC), the dependence of α can be expressed as $\alpha = \alpha^o + 0.20 \cdot FC$, where α^o refers to the pH- and temperature-dependent yield of O, while the second term reflects the dependence of the relative yield of O on the FC. This latter term should depend on the former, since if more molecules formed O due to pH or temperature, fewer molecules may be converted into O via cooperativity.

At present it seems premature to suggest a model for the mechanism of the cooperative interaction expected to regulate additionally the relative yield of O. However, some expectations can be defined: As the dichroic ratio of O is in good agreement with that of M_f, it appears to be probable that the O generated by the additional cooperation is formed isotropically in the M_f population. Thus, a statistically acting cooperation seems to be more reasonable. Moreover, the appearance of the O intermediate is associated with retarded proton release (from Asp-85) on the extracellular side of the purple membrane at relatively high proton concentrations (see e.g. [1]). Thus, it is probable that the additional cooperation regulating the yield of O acts on this side of the purple membrane, in the opposite manner to the cooperativity regulating the M decay, which is known to cause [8] significant changes in the BR structure on the opposite (cytoplasmic) side (at least around Asp-96).

In a recent paper [13], on the basis of kinetic considerations, Hendler forwarded the idea according to which only M_f would be involved in the accumulation of O. Although his basic conclusion (that we argued in the same paper) that O is formed only from M_f is confirmed by the present study, this statement turned out to be rather incomplete, due to the complications which arise when one tries to correlate the maximum amount of O and M_f at different exciting flash intensities.

The final conclusion of the present communication is that the yields of all the intermediates present in the millisecond time domain of the BR photocycle depend on the fraction cycling. Moreover, the present data also suggest that the formation of O is probably in connection with a cooperative event other than that regulating the M intermediate decay, and that in a model with two different parallel M intermediates only the rapidly decaying M may form O. Finally, as an extension of our model describing the light intensity dependence of the photocycle with the O intermediate such a scheme can be suggested, where the M_f/M_s ratio is regulated by a cooperativity acting on the cytoplasmic side, and the O/N ratio is regulated besides the pH and temperature by another cooperativity acting on the extracellular side:

$M_f \rightarrow O \rightarrow BR$

$M_f \rightarrow N \rightarrow BR$

$M_s \longrightarrow BR$

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