Actinic Light Density Dependence of the Bacteriorhodopsin Photocycle

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ABSTRACT The photocycle of bacteriorhodopsin (BR) was studied in the 0.3 μs to 10 s time interval after excitation, using a wide range of actinic light intensities (10 ns half-duration, 0.06–60 mJ/cm^2), at neutral and alkaline pH values. The relative weights of the rapidly and the slowly decaying components of the M intermediate (M_f and M_s, respectively) and the yield of the third millisecond component, N(R,P), are the function of the exciting light intensity (density), while their lifetimes are not. The relative weight of M_s is found to be a linear function of the portion of the BR molecules undergoing the photocycle. This suggests the existence of a cooperative interaction of the BR molecules arranged in the crystalline purple membrane sheets. Another source of M_s is also found, which results a nonvanishing relative weight of M_s even at very weak actinic light density values. The explanation for this may be a branching, or the heterogeneity of BR itself or with its environment. It is shown that the relative weights of the rising and decaying components of the M form(s) do not correlate directly with each other.

INTRODUCTION

Bacteriorhodopsin (BR) is the simplest known biological light energy transducing molecule (for a recent review see Refs. 1 and 2). The photocycle of the all-*trans* retinal containing BR (light adapted BR, LA-BR) is the description of the spectroscopically observable cyclic changes after photoexcitation. The BR's function is light-driven proton pumping in halobacteria. The molecular basis for this function is to be found in the changes in the proton affinity of the Schiff base and various protein residues around it that move protons from one surface to the other. In the first approximation, but only under fairly restricted external conditions (pH 6–7, 20–30°C, low ionic strength, etc.) the unidirectional, unbranched BR photocycle models (3, 4) describe the main transient absorption changes occurring in LA-BR after light excitation. However, besides the BR photocycle intermediates (K, L, M, N, O) originally proposed, the existence of several new intermediates, J, K′, L′, M_{fast} (M_f), M_{slow} (M_s), N(R,P) have been suggested (5–13). It was shown by Nagle et al. (14) long ago that the original photocycle model (4) cannot account for many experimental findings. They also summarized the most important propositions to be considered in the creation of photocycle schemes.

Many attempts have been made to interpret the new data, with the introduction of different branchings, reversible steps, and new intermediates in the photocycle models (for reviews see Refs. 9, 15, 16).

On the basis of new kinetic, low-temperature absorption and Raman spectroscopic studies, the heterogeneity of LA-BR (6, 17, 18) and thus independent BR photocycles have been recently proposed (5, 7, 18, 19, 20). At the same time the possibility of a cooperative interaction between the BR molecules arranged in trimers (e.g., at the level of the M intermediates) (21, 22), has been almost completely forgotten or ruled out (10, 16, 23).

However, there is much recent evidence for the influence of the exciting light intensity on the BR photocycle. It was reported that the yield of the N intermediate decreases with increasing light intensity (11). The decay of the M intermediate is affected by the exciting flash intensity (24). On the basis of the light-induced conductivity change measurements, cooperativity (long distance, exceeding the size of the trimers) of the BR molecules has been proposed (25).

As an alternative or additional explanation for the complexity of the photochemistry of BR, the biphasic decay of the absorption changes detected at 412 nm (δA_{412}) has recently been assigned to reversible reactions between M and O, or M and N (23, 26–28) instead of assuming two separate, different M intermediates arising from two different forms of BR.

The existence of the actinic light density dependence of the LA-BR photocycle was shown recently by us, in experiments proving the existence of either the light-induced heterogeneity of the BR ground state or a cooperative interaction between the BR molecules (29). In the present study we deal with this problem in detail.

MATERIALS AND METHODS

Purple membranes (PMs) were isolated according to standard procedures. In order to avoid the contribution of the light-scattering signals to the absorption changes (30) and the distortion of the signals by the rotation of the PMs (31), the PMs were embedded into 10% polyacrylamide gel (32).

The samples, 1-mm-thick slices of polyacrylamide gel with PMs (0.24 absorbance at 570 nm for 1 mm) were soaked and rinsed several times in 30 mM universal buffer (0.6 citric acid, 0.39 monopotassium sulfate, 0.18 borate, 0.5 diethylbarbiturate, w/w%) at the pH indicated, with 1 M NaCl.

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Abbreviations used: BR, bacteriorhodopsin; M_f and M_s, fast and slow components in the decay of the M intermediate; *p*, proportion of the BR molecules undergoing the photocycle (fraction cycling); PM, purple membrane; δA, transient change in the absorption of BR at the wavelength of the measuring beam given in the index.

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The measurements were carried out in a 2-mm-thick spectroscopic quartz cuvette, thermostated at 20° or 30°C.

The transient absorption changes (δAs) were measured in an absorption kinetic measuring system as follows. The monitoring beam was obtained from a 75-W xenon arc lamp (PTI Inc., PS 200X, Princeton, NJ) with an optical feedback stabilization. One of two identical monochromators (Oriel, 7240 series, Stamford, CT) was in front of and the other behind the sample (spectral width ~4 nm). The light intensity changes were detected by a photomultiplier tube (Hamamatsu R928, Hamamatsu Photonics K. K., Hamamatsu City, Japan), which was additionally protected by cutoff filters.

The signal was amplified by a home-built preamplifier (~0.3 μs response time) and collected by a logarithmic time-based transient recorder (33). In the first ~12 μs after the exciting flash, the signal was measured and stored on a linear time scale (with 0.1 μs sampling rate). After the first ~12 μs, the data points were collected, with a 2 μs sampling rate during the whole measurement. The data collected in each 2-μs segment were averaged over logarithmically increasing time periods. So that 30 data points were stored and resolved in each time decade. In this way the signal-to-noise ratio significantly increased with the increasing time elapsed after the exciting flash. The data were averaged and converted to absorption changes in a ZX Spectrum microcomputer (Sinclair Research Ltd., Cambridge, UK) and stored and processed with a personal computer (Olivetti M24). The data traces were analyzed by an exponential fitting program (written by Dr. Cs. Bagyinka, based on the least squares method).

For excitation, an excimer laser (JATE, Hungary) was used (~10 ns pulse half-width) with a dye laser option (Coumarin LC 5000, emission maximum at 505 nm; Lambda Physik, Göttingen, Germany). The 505 nm actinic wavelength was chosen to have a minimal excitation of the K intermediate. Since the absorption of K at 505 nm is still significant, the excitation of K at high laser intensities is unavoidable. However, as we showed previously (29) with respect to the phenomena discussed in this paper, the excitation of K is not of importance. In our experiments we used the homogeneous part of the exciting excimer laser spot. Moreover, in separate control experiments, when Nd-YAG and flash lamp-pumped dye lasers were used, where the laser spot was highly homogeneous and even a beam scrambler was also applied, the light intensity dependence of the BR photocycyle was exactly the same as we present here.

The diameter of the measuring beam spot on the cuvette was 3 mm. The actinic laser spot was applied quasi-coaxially (under ~20°) and it had a diameter of 4 mm. The light adapting continuous wave argon laser was applied (also under 20°) from the other side. The planes of polarization of the monitoring and the exciting beams were set at the "magic angle" 57.5° (16).

In order to exclude the possible exciting effect of the measuring beam, its intensity was carefully reduced at each wavelength and its pH was studied, until, at a reduction of fivefold, it did not affect the kinetic properties of the signal at all.

All measurements were carried out on completely light-adapted BR. In order to maintain the light-adapted state of BR, especially during the long measurements at low actinic light densities (when 200–1000 traces were averaged with a repetition rate of 0.05–0.5 Hz, depending on the pH and the temperature), each fifth exciting flash was followed by a 2–5-s light adaptation period (light of 514 nm, 20 mW/cm^2 from a continuous Argon laser, ILA-120; Carl Zeiss, Jena, Germany). The light adaptation was followed by a 6–15-s dark period, to ensure the complete decay of the BR molecules from a photostationary state. It was important that we avoid the necessity of the presence of a weak continuous side illumination (16). We found that at alkaline pH values even the 1.6 mW/cm^2 intensity of 514 nm continuous or periodic side illumination causes significant build-up of the late intermediates of the BR photocycle into a steady state, and their complete decay requires much more than 400 ms (mentioned in Ref. 16), especially above pH 8.5.

In order to completely exclude the possibility of the reexcitation of the BR intermediates by the consecutive exciting flashes, the time intervals between the flashes were always at least five times longer than the lifetime of the longest living intermediate.

In control experiments almost all of the experimental conditions (salt concentration, pH, temperature, exciting wavelength, etc.) were altered to ensure the generality of our findings.

Solubilization of the PMs was carried out by Triton X-100 at room temperature, for 24 h, with a protein:detergent ratio of 1:4 (w/w), in 7 mM phosphate buffer, at pH 6.9.

Partial bleaching of the sample by hydroxylamine treatment was done by the method described in (34).

The proportion of the BR molecules undergoing the photocycle (p) was calculated from $p = k \cdot dA_{412nm}/A_{570nm}$, where A_{570nm} is the absorption of the sample at 570 nm, dA_{412nm} is the amplitude of the absorption changes at 412 nm, and k is the ratio of the absorption changes at 570 and 412 nm ($k = 2$) (22).

RESULTS

Overview of the characteristic absorption changes accompanying the BR photocycle

The separation of the BR photocycle intermediates, which strongly overlap in their absorption and in the time domains of their appearance and decay, is a difficult job. In some studies (e.g., Refs. 16, 28, 35) the simultaneous, multiexponential fit approach to the data, taken at numerous wavelengths, was used. Recently, we succeeded in the separation of the amplitude spectra of M_f, M_s, and N(R,P) (19). The amplitude spectra of these late kinetic components of the BR photocycle seem to have different isobestic points, relative absorption amplitudes, and lifetimes. These three components with three other, faster ones (which are often referred to as (the decay of) K, L_f, and L_s in the literature) (18), in the microsecond time domain, describe the photocycle of BR to a good approximation and account for the results presented in this paper. At neutral pH it is mostly the O intermediate that accumulates rather than N.

The absorption changes (δA) occurring during the BR photocycle, at the selected wavelengths, are shown in Fig. 1. The first part of the data traces were measured on a linear, the second part on a logarithmic time scale (see Materials and Methods). (The reasoning for the following tentative component assignments is given later.)

The absorption change at 335 nm (δA_{335}) (Fig. 1, *trace 1*) has a biphasic rise, where the dominating first part remains unresolved under our measuring conditions. In the decaying part of the signal there is no significant contribution of M_f, and that is why only M_s and N can be found (19), allowing us to study the relation between them.

The absorption kinetics at 412 nm (Fig. 1, *trace 2*) have no unresolved fast component. At this wavelength the absorption increase consists of two components, which are associated (e.g., in Ref. 18) with the decay of the L_f and the L_s intermediates (i.e., with the accumulation of the M intermediate(s)). The decay at this wavelength consists of mainly the M_f and M_s components. The contribution of N to the δA_{412} signal is small even at high pH (19).

The bleaching signal of BR during the photocycle can be observed at 570 nm (Fig. 1, *trace 3*). In the 1–100-μs time range the decay of L_f and L_s can be found, indicating L to M transitions. In the disappearing part of the signal all of the

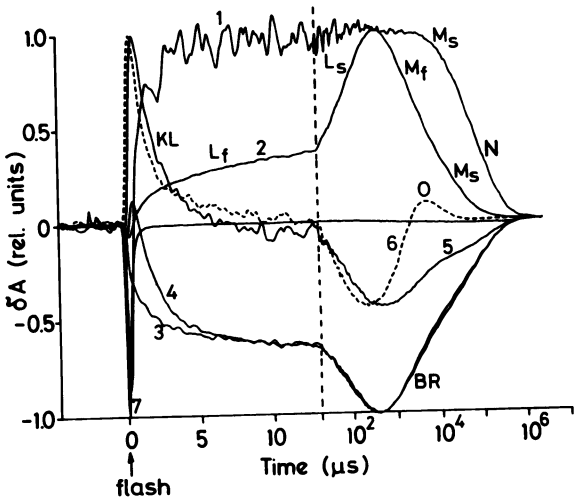

FIGURE 1 The absorption changes (normalized at their maxima or minima) accompanying the LA-BR photocycle are shown (in 1 M NaCl and at ~6 mJ/cm² (0.7 photon/BR) actinic light energy density at 505 nm) at some characteristic measuring wavelengths: *1*, 335; *2*, 412; *3*, 570; *4*, 610; *5*, 650 (at 20°C, pH 8.25); *6*, 650 (*dotted trace*, pH 7.6, 30°C) nm. The vertical dashed line shows the beginning of the logarithmic time scale of the data collection. The components dominating in the time domain at the given wavelength are also indicated. Trace 7 shows the time resolution of the system, where the electrical and the light noise of the laser pulse was recorded in the absence of the measuring beam. At pH 7.6 there is a pronounced absorption increase at 650 nm (trace 6) due to the accumulation of the O intermediate, while it is missing at pH 8.25 (trace 5).

late kinetic components (M_f, M_s, and N) contribute at alkaline pH values (19). At nearly neutral pH values only a residual amount of N can be found (36), but the presence of the O intermediate is significant (with a lifetime nearly equal to that of M_s). The absorption kinetic signal at 610 nm (Fig. 1, *trace 4*) seems to be very similar to the δA_{570}, except that the KL to L transition (37, 38) in the 1–10-μs time range contributes significantly to it.

For the completeness of the illustration of the photocycle, the δA_{650} is shown in Fig. 1 (*traces 5* and *6*). In the rising part of the traces besides the two L components, the decay of the KL can also be seen (~1 μs lifetime). In the decaying parts of the traces, either O at neutral pH or N at high pH seems to contribute considerably, beside the decay of M_f.

The dependence of the kinetic components of the BR photocycle on the light energy density of the actinic flash

In most of the recent absorption kinetic studies on BR no special attention was paid to the actinic light density used in the experiments. When it was checked, usually no dependence was found (10, 16, 23). This could be explained by the following factors: the narrow range within which the light intensity was varied; the relatively modest signal/noise ratio; the short time of the measurement (e.g., at high pH it is very difficult to measure the M decay on a linear time scale).

In contrast we found a drastic change in the absorption kinetics of BR when the energy density of the exciting flash was varied over a wide range (above 100-fold) (29). It was confirmed recently in Ref. 24.

In Fig. 2 the actinic light density dependence of the absorption changes of BR is shown at pH 7.6, 8.2, and 9.5, measured at 412 nm. The δA_{412} traces (normalized at their maxima) show that the L_f and the L_s components (the rising parts of the signals) are independent, while the decays of the late kinetic components are very much influenced by the intensity of the exciting light. This fact provides direct evidence for the decoupling of the rising components of δA_{412} from the decaying ones noted in (20). On one hand the dependence of the kinetics on the actinic light density is similar at pH 7.6 and 9.5. On the other hand at each actinic light density studied, the increase in the weight of L_f and the increase in the lifetime of M_s with increasing the pH can be seen (Figs. 2 and 3 and Table 1), as expected from the literature (e.g., Refs. 17, 39, 40).

The decay phase of the data traces is shown in Fig. 3 at three informative wavelengths (335, 412, and 570 nm), on an expanded time scale. The rising parts were omitted, as they are only slightly influenced, if at all, by the actinic light density. Qualitatively the influence of the light density is the

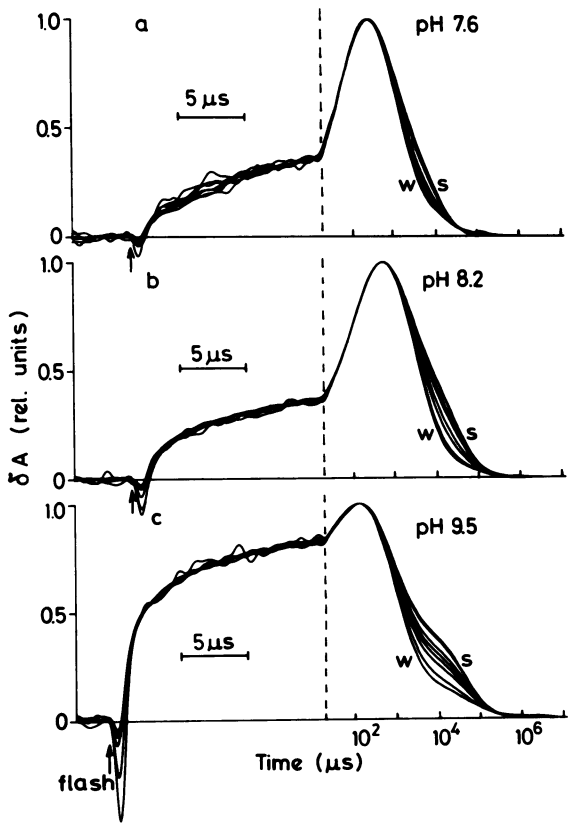

FIGURE 2 The dependence of the normalized absorption changes at 412 nm on the energy density of the exciting laser flash (varied between ~0.24 and 60 mJ/cm² (0.03–7.5 photon/BR) at 505 nm) is shown at pH 7.6 (30°C) (*a*), pH 8.25 (20°C) (*b*), and pH 9.5 (30°C) (*c*) in 1 M NaCl. The rising parts of the traces (including the decays of L_f and L_s) are independent, whereas in the decay the relative weight of M_s is increasing with increasing actinic light density (*w*, weakest; *s*, strongest exciting flash).

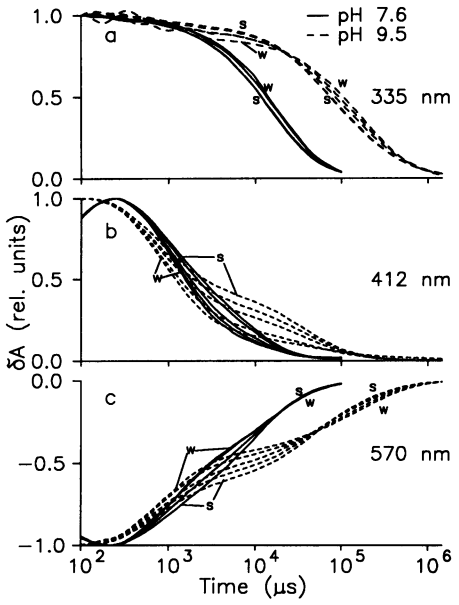

FIGURE 3 The decaying parts of the normalized absorption changes measured at different actinic light energy densities are plotted on an expanded time scale. The data were measured at 335, 412, and 570 nm, at pH 7.6 (*solid lines*) and pH 9.5 (*dashed lines*) at 30°C in 1 M NaCl. (The actinic flash was varied as in Fig. 2.) The increasing energy density of the actinic light causes an apparent acceleration at 335 nm (components: M_s and N); deceleration at 412 nm (components: M_f and M_s); and the combination of these two effects at 570 nm (components: M_f, M_s, and N) manifesting in different time domains. The apparent changes in the decay kinetics are due to the shifts in the ratios of M_f, M_s, and N. (See Fig. 4, Table 1, and text.)

TABLE 1 The relative weight of M_s at weak and strong excitation, and the lifetimes of the millisecond kinetic components at different pH values

pH		7.0	7.6	8.2	9.5
Temperature		30	30	20	30
Relative weight of M_s	W*	13	17	15	15
	S	45	44	47	44
Lifetimes of the components (ms)	M_f	1.0	1.3	3	1.0
	M_s	6.0	13	28	55
	N	—	45	200	400
Figures‡		5	2, 3	2, 4	2, 3

*W, weak; S, strong.
‡The measured data or their evaluation are shown in the referred figures.

same at pH 7.6 as at pH 9.5, except that the changes in the shape of the δA traces are seemingly more pronounced at higher pH, due to the larger differences in the lifetimes of M_f, M_s, and N. This finding is clearly shown in Table 1. The lifetimes (see Table 1) were independent of the actinic light density (see below). The increasing actinic light density apparently speeds up the last part of the δA_{335} signal (by decreasing the contribution of N; Figs. 3*a* and 4*a*). But at 412 nm it seemingly has an opposite effect. However, note that according to the time constants and the amplitude spectra (19), the slow component of the δA_{412} decay is attributed mainly to the decay of M_s, not to that of N. The data presented here give independent evidence for this, because if the N intermediate contributed to the δA_{412} considerably, the last (slowest) parts of the traces should cross each other, as in the δA_{335} and δA_{570} data. (The slight, apparent slowing down of the first part of δA_{335} also belongs to the decay of M_s (Fig. 3*a*, between ~100 μs and 20 ms)). Consequently, even at high pH (where only a very small amount, if any, of O is formed) in the millisecond time domain at least three exponentials are required for describing the absorption changes. However, in some recent photocycle models (proposed, e.g., in Refs. 41 and 23) the late part of the photocycle is discussed as $M_2 \leftrightarrow N \rightarrow BR$. This means that only two intermediates (and thus two apparent rate constants) would be involved in the late part of the photocycle. Our data discussed here can be described only with three kinetic components.

The opposite effect of the increasing actinic light density on M_s and N can also be clearly seen in the decay of the depletion signal of BR, measured at 570 nm (Fig. 3*c*), especially at pH 9.5, where the relative yield of N is significantly enhanced (42). At high pH the lifetimes of M_f, M_s, and N differ from each other by more than 10 times, so they can be found in different time domains. Between ~1 and 50 ms the decay of the M_f and M_s components can be seen, while at ~100 ms after the exciting flash, the recovery of BR from N becomes the dominating spectral change. In conclusion, one can state that the effect on M_s of increasing the actinic light density is the opposite of the effect on M_f and N.

The number of components used for the satisfactory description of a given absorption kinetic trace is always a controversial issue and depends on the type of the model used. It is not the goal of this present study to determine the number of BR photocycle intermediates or to propose a model. We restrict our analysis and discussion to a minimum number of kinetic components, even when they are widely accepted as distinct intermediates (e.g., N).

In this paper we are dealing mainly with reactions occurring in the millisecond time domain, that is, the decay of the M type and N components. Below we give briefly the reasoning for the number of exponentials fitted to absorption changes at different wavelengths. We chose the data measured at pH 9.5 (Fig. 3, *dashed lines*), where the rates of the different processes are more separated. In most of the cases these exponentials can even be distinguished visually (43). We start with the description of δA_{412} (Fig. 3*b*). It is obvious that the shape of the traces changes strongly with changing exciting light intensity. This fact by itself indicates the necessity of fitting at least two exponentials. These two exponential components are affected differently by the exciting light density and pH. We call them M_f and M_s. At 335 nm (Fig. 3*a*) the traces measured at different light intensities cross each other, which also indicates that at least two exponential components are present. Around 10 ms the M_s and after that the decay of N can be seen. At 570 nm in the bleaching signal of BR (Fig. 3*c*) all three of these components can be seen: M_f and M_s in the ~0.5–50-ms time domain (as in Fig. 3*b*), and N is showing the same crossover (as in Fig. 3*a*). It is important to note that the rates found for these components at different wavelengths were similar (Fig. 4 and Table 2). All of the qualitative descriptions given above for

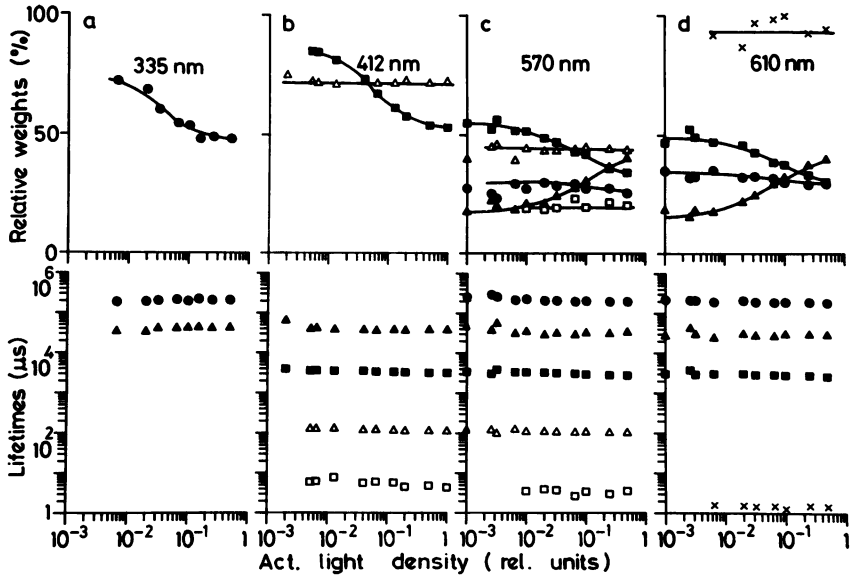

FIGURE 4 The results of exponential fits to the absorption changes measured at four wavelengths, in a wide range of exciting flash energy densities are shown (pH 8.25, 20°C, 1 M NaCl). (The actinic light density at 1 relative unit was ~60 mJ/cm^2 (7.5 photon/BR).) The relative weights of the decay of M_f and N are decreasing with increasing the actinic light density (*a* and *b*, respectively). The relative weights of KL, L_f and L_s decays are not influenced by the actinic light density. The lifetimes of the components are independent from the actinic light density and the wavelength of the measuring beam. The same rates have been found for the same components measured at different wavelengths (see also Table 2). The fitted components are: δA_{335}, decay, M_s, N; δA_{412}, rise, L_f, L_s, and decay, M_f, M_s; δA_{570}, rise (from 3 μs), L_f, L_s, and decay, M_f, M_s, N; δA_{610} is similar to δA_{570} and has a one-exponential fit to the KL in the first 3 μs. The relative weights of the rising components were calculated by taking the sum of the amplitudes of the decaying components as 100% at the given wavelengths. The assignment of the symbols corresponding to the kinetic components of the BR photocycle is as follows: ×, KL; □, L_f; △, L_s; ■, M_f; ▲, M_s; ●, N.

TABLE 2 The lifetimes (with their SDs) of the components shown in Fig. 4

Wavelength	$\tau(L_s)$ (μs)	$\tau(M_f)$ (ms)	$\tau(M_s)$ (ms)	$\tau(N)$ (ms)
330 nm	—	—	40.8 ± 3.9	200 ± 12
412 nm	130 ± 5	3.39 ± 0.23	39.0 ± 1.6	—
570 nm	118 ± 5	2.96 ± 0.27	31.8 ± 2.6	206 ± 11
610 nm	116 ± 10	2.88 ± 0.14	29.3 ± 2.1	196 ± 10
Global*	122 ± 11	3.12 ± 0.33	34.9 ± 5.4	201 ± 11

*These values are calculated from all the data shown in Fig. 4 at the different wavelengths and actinic light densities.

the data at pH 9.5 are valid also for the data measured at pH 7.6 (Fig. 3*a–c, solid lines*).

The fact that the present data indicate the existence of at least three millisecond components at high pH is an argument against the currently frequently used model of the BR photocycle (see, e.g., Ref. 23) suggesting an M ↔ N → BR scheme of the M decay and BR recovery. At high pH, where the M → N and N → BR reactions are well separated in time, and the O intermediate does not accumulate, this scheme predicts two apparent lifetimes for the millisecond part of the photocycle: the first one would be due to the equilibration of M and N, and the second one would represent the decay of this equilibrium mixture to BR. As a consequence, the ratio of the 412 and 570 nm absorption changes should reflect only the settling of the equilibrium, and it should then remain constant until the complete recovery of BR. On the contrary, this ratio has a more complex time dependence (the plot is not shown) due to the presence of the third millisecond component (i.e., the M_s) discussed above. This is an additional argument against the explanation of the biphasic decay of the M intermediate with the backreaction between N and M. Independent evidence has been shown previously for the existence of three components in the millisecond part of the BR photocycle at high pH (13).

The relative weights and the time constants of the exponentials (according to the components shown in Fig. 1) fitted to the data set measured at pH 8.25 were plotted versus the exciting light density in Fig. 4 (*top panels*). Very close lifetimes were found for the components at different measuring wavelengths, proving the self-consistency of the evaluation (Fig. 4, *bottom panels*, and Table 2).

Fig. 4 and Table 2 show that the lifetimes are independent of the actinic light density within 3–10%. The deviations of the lifetimes determined at different wavelengths (5–30%) should also be considered small, taking into account the complexity of the system analyzed.

For the clarity of the description of the basic phenomena of the light density dependence of the BR photocycle the absorption kinetic traces δA_{335} and δA_{412} were fitted with only two exponentials, since the third component had a relative weight of less than 5% (13). This simplification does not have an influence on our conclusions presented in this paper, but it could cause the slight differences in the lifetimes determined at different wavelengths.

The relative weights of M_f and N (Fig. 4, *top panel*) decrease versus the actinic light density. This effect is obvious

from the 335 and 412 nm measurements but can also be seen at 570 and 610 nm. From the fact that the same tendency appears in the experiment measured at 545 nm (data not shown), where the depletion signal of the 13-*cis* and all-*trans* retinal-containing BRs have an isobestic point (44), we can conclude that a possible change in the isomeric ratio of the retinal is not responsible for the actinic light density dependence. The relative weights of KL, L_f, and L_s are independent of the actinic light density. The rates of all of the components remain virtually constant upon changing the intensity of the exciting flash more than 100-fold at all of the six measuring wavelengths (the 545 and 650 nm data are not shown) (see Fig. 4, *bottom panel*). The apparent changes in the decay kinetics observed in Figs. 2 and 3 are due to the shifts in the ratios of the components formed at different actinic light densities, as summarized in Table 1.

The same effects of the exciting flash have been found at low ionic strength; only the ratio of M_f and M_s were slightly different, and the decay of M_s was ~2–3 times faster than in 1 M NaCl. The actinic light density dependence exists in other buffers (e.g., phosphate), at other exciting wavelengths (e.g., at 590 nm), in distilled water suspension (i.e., not immobilized in gel) of the purple membranes, and at any polarizations of the actinic and measuring beams (data not shown).

In contrast, actinic light density dependence was not found in samples monomerized by Triton X-100 treatment, as also shown by others (24). The yield of the light intensity-dependent M_s decreased if the average distance of the functionable BR molecules was increased by partial bleaching of the sample by hydroxylamine. Bleaching 80% of the BR molecules resulted in an almost complete disappearance of the actinic light density dependence of the M kinetics at 412 nm (data not shown).

Yields of M_f and M_s versus actinic light density

The relative weight of M_s versus the actinic light density at pH 7 is shown in Fig. 5*a*. There is a drastic increase in the relative weight of M_s from ~15 to 45% with increasing actinic light density.

The amplitudes of M_f and M_s and their sum (which is nearly equal to the total signal amplitude at 412 nm) versus the actinic light density are shown in Fig. 5*b*. According to the literature (45) this sum of the amplitudes cannot be described as a simple exponential saturation curve. The BR molecules are immobilized in the purple membrane sheets; thus they cannot tumble rapidly during the excitation. This leads to a retarded saturation of the signal amplitude versus the flash intensity. The mathematical formalism of this function of the saturation can be expressed in terms of the error functions (see, e.g., Ref. 46), as it has been shown in Ref. 45. This theory applied to our data results the solid line shown in Fig. 5*b*, where the agreement with the data is excellent.

However, the decay of the δA_{412} cannot be described as a simple first-order reaction, since the kinetic traces taken at different actinic light densities have very different shapes due to the shift in the relative weights of M_f and M_s (Fig. 5*a*) under all conditions studied in the present work (pH 7–10, 0–30°C, 0 and 3 M NaCl, with excitation wavelengths of 505, 532 and 590 nm; data not shown). Consequently, in our opinion, the simple readout of the maxima of the absorption changes (according to Ref. 45) do not give the complete description of the saturation curves of BR. At least the fitting of two exponentials is necessary to describe the observed decay of δA_{412}.

In the very weak actinic light density range, the amplitudes of both of the M_f and M_s components are linearly proportional to the actinic light density used. (The initial slopes of the saturation curves are 1 in the log-log plots; see Fig. 5*b*.) In this actinic light density range (below ~0.2 mJ/cm^2) the ratio of M_s to the total M remains approximately constant, ~13% at pH 7.1 and 30°C. Upon increasing the actinic light density the yield of M_f declines from the linear dependence, that is, it starts to saturate, while the yield of M_s remains apparently linear.

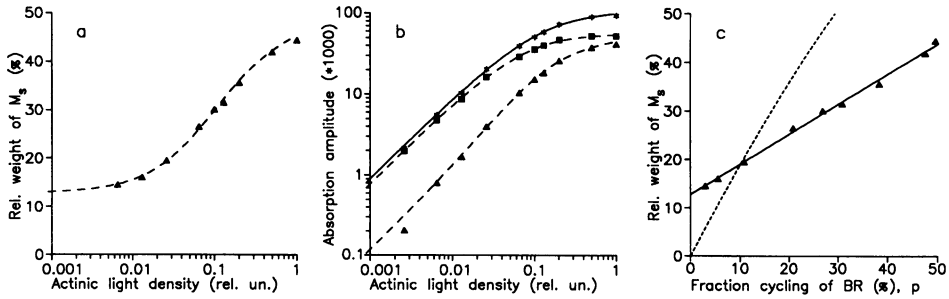

FIGURE 5 Three different representations of the decay of the δA_{412}. The data were obtained from the same dataset. (*a*) The relative weight of M_s (▲) versus the actinic light density. (*b*) The saturation of the amplitudes of M_f (■), M_s (▲), and their sum (*) versus the actinic light density. (*c*) The relative weight of M_s (▲) versus the fraction cycling (*p*). The lines in the figure are computed as follows. In *b* the solid line is calculated according to the theory of the saturation (45). Replotting *a* versus *p* resulted in a linear dependence of M_s (*c*; the *solid line* was obtained by fitting a linear regression function to the data points). Using the linear dependence of M_s on *p* the saturation curves of the absolute amplitudes of M_f and M_s can easily be computed (*b, dashed lines*). After this step the saturation of the relative weight of M_s can be obtained from *c* and *b* (*a, dashed line*). The dotted line in *c* is the function proposed by Ohno et al. (22). The precision of the relative value of *p* is ~1–3%; however, due to technical reasons its absolute value has an uncertainty of ±10%. Measuring conditions: pH 7, 30°C, 1 M NaCl, excitation ~0–60 mJ/cm^2, 505 nm. The lifetimes of M_f and M_s were 1 and 6 ms, respectively. The absorption of the sample was 0.38 at 570 nm. The relative plans of polarization of the measuring and the exciting beams was set at the "magic angle."

By further increasing in the energy density of the exciting flash, M_s also starts saturating, approximately above 2–4 mJ/cm^2.

Even at our highest actinic light densities (~60 mJ/cm^2) BR had only reversible changes. The amplitudes and the shapes of the traces did not depend on the number of repetitions, and by decreasing the actinic light density back to the linear dependence region the same curve was obtained. This fact indicates that the photodestruction described recently (47, 48) cannot be responsible for the actinic light density dependence found. Otherwise, the actinic light density region, in which the destructive processes may start, is above five photons per BR (48), while the kinetic changes presented here become considerable far below this value.

From the different intensities at which M_f and M_s saturate, it is obvious that any study on the dependence (e.g., pH, salt, temperature) of their ratios is influenced by the energy density of the actinic flash, unless the actinic light density is kept at a fixed value or unless the experiment is carried out at extremely low actinic light densities.

Correlation between the slower phase of the M decay and the fraction of the BR molecules undergoing the photocycle

The relative weight of M_s ($M_s/(M_f + M_s)$) versus the portion of molecules undergoing the photocycle (p) is shown in Fig. 5*c* (*solid line*). This is a linear function, and thus the yield of M_s is a quadratic function of p (as here we assume that p is proportional to $M_f + M_s$, the total amount of M formed). p is defined according to the method of Ohno et al. (22), and their prediction for the relative weight of M_s is shown by a dotted line.

The relative weight of M_s does not become zero. The extrapolated value of the relative weight of M_s at zero actinic light density is ~13% (Fig. 5*c*). This is in agreement with the range of 5–20% given for this quantity by Kouyama et al. (12).

The linear dependence of the relative weight of M_s on p (along with the control experiments carried out on monomerized and on partially bleached samples) suggests kinetic cooperativity between the neighboring BR molecules, for similar reasons, as has been concluded previously (22, 24).

On the other hand, our results differ considerably from the description (indicated by a *dotted line* in Fig. 5*c*) suggested by Ohno et al. (22):

I. The relative weight of M_f was expressed there as $(1 - p)^2$. From this expression, at not too strong excitations, p^2 can be neglected, and hence the slope of the relative weight of M_f should be about −2 (and that of M_s, 2). The slope of the relative weight of M_s, we find, is about 0.6 versus p (Fig. 5*c*). This difference between that theory and our measurements indicates that the cooperative mechanism has to be different from the suggested one (22). The theory suggested by Ohno et al. (22) assumed that (*a*) if there are two molecules excited in a trimer, both of them turn into M_s, and (*b*) an excited molecule has two possible neighbors (in the trimer), which may turn it into M_s, if at least one of these two neighbors is excited also.

The difference between the theory and our measurements (Fig. 5*c*, *dotted* and *solid lines*) indicates that either cooperativity has less efficiency in turning the cycling molecules into M_s or the interactive units of cooperativity are different from the trimers of BR (as has been suggested in Ref. 25).

II. M_s does not vanish. It has a considerable contribution (~13%) even when the actinic light density is very small. This component is not due to a residual contribution of N to the absorption changes at 412 nm, because M_s and N have considerably different lifetimes at high pH; and at neutral pH, when N does not accumulate, the decay of M remains biphasic.

DISCUSSION

There are very few data and no comprehensive studies in the literature on the dependence of the BR photocycle on the density of the exciting light. When the decay of the δA_{412} from different photostationary levels was studied (21) at −20°C, the rate constants of the biphasic decay correlated with the amount of the M intermediates. In our present fast spectroscopic kinetic study we found that the decay rates of the components of the M intermediate are independent of the exciting light intensity.

In another study (22) the yield of M_s was found to be proportional to the square of the flash intensity, and the lifetimes were found to be independent. In that experiment, carried out at high pH and at lower temperature (~9°C), the 260-μs-long flash also could excite several early intermediates of the BR photocycle. This may account for the different slopes of the relative weight of M_s (versus p) between our experiments and those of Ohno et al.

We found that the yield of M_s is linearly proportional to the flash intensity even under conditions similar to those of Ohno et al. (22) (at pH 9.8, 8°C, but for excitation we used a 10-ns-long laser pulse; data not shown), if the excitation is weak enough, where according to Ohno et al. no significant M_s should be formed. The clear biphasic decay of the M intermediate(s) at weak exciting light intensity may originate in branching or in some kind of heterogeneity (previously found, e.g., in Ref. 39 and referred to in Ref. 22) of the BR molecules arranged in the purple membrane sheets. Kouyama et al. (12) suggested that the nonzero relative weight of M_s at low light intensities may originate in a back-reaction between N and BR ranging between 5 and 20% depending on the solvent composition. At this point none of them can be excluded.

Drachev et al. (11) found that low light intensity is a favorable condition for the formation of the N intermediate. This fact is confirmed by our present results if the slowest kinetic component (N) in our evaluation describes the decay of the N intermediate.

In some recent studies the influence of the flash intensity on the photocycle was checked, and no dependence was concluded. In a multi-wavelength absorption kinetic study (16)

as control, the fraction of the BR molecules cycling was decreased from 19% to 9%, and a factor of 4 was expected in the amplitude of a component, if it originates in the cooperativity of the BR molecules (16). In contrast, on the basis of our measurements presented here the relative weight of M_s is expected to decrease from ~23% to 18% (see Fig. 5*c*), if the fraction cycling is decreased from 19% to 9%. This change is drastically smaller than was expected in the study of Xie et al. (16), and they might have not been resolved. In the other study concluding that there is no actinic light density dependence in the BR photocycle kinetics (23), the fraction cycling was below 10%, and the kinetics of the photocycle were measured by an optical multichannel analyzer, point by point. In our opinion, in such experimental conditions the observation of a dependence on the actinic light density is almost impossible, as the expected changes in the kinetics are rather small, and in point-by-point measurements the uncertainties of the data points seem to be comparable with these changes.

Two forms of the BR ground state have been demonstrated recently (18) on the basis of their different absorption spectra and photoreactions, suggesting that at alkaline pH BR is transformed into an acidic state. However, this process is reflected by the first part of the BR photocycle, and in the second part of the BR photocycle we cannot find any process that correlates with the suggested transition (that can be monitored according to the method of Ref. 18, e.g., by the shift in the ratios of L_f and L_s).

Another study relevant to our present work was published by Nagle et al. (45). In that work the theory of the saturation of BR was developed in detail. They compared their experimental data to the theoretical curves, and they used only the simple overall amplitude of the absorption change. However, if the decay of the absorption change is not a single exponential, but, for example, the sum of two exponentials, the overall amplitude might not reflect the different features of the individual components. In fact, when we plotted the overall amplitudes of the δA_{412} versus the actinic light density, the theoretical curve proposed by Nagle et al. (45) fitted very well to the data points (Fig. 5*b*, *solid line*) but was not suitable for the proper description of the components (M_f and M_s), separated by exponential fitting. This possibility was predicted by Nagle et al. (45) where a cooperative interaction is involved in the BR photocycle. Since our data suggest a cooperative interaction between the BR molecules we extended the application of the study by Nagle et al. (45) as follows.

The shapes of the saturations of M_f and M_s shown in Fig. 5*b* (*dashed lines*) are the result of the linearly increasing yield of M_s and the saturation of the yield of M versus the flash intensity previously described in (45) (Fig. 5*b*, *solid line*). The increase in the relative weight of M_s at higher light intensities shifts the saturation of M_s toward the higher light intensities, as shown in Fig. 5*b* by a dashed line. (The expected saturation of M_f is also shown in Fig. 5*b* by another dashed line.) In spite of the presence of a cooperative interaction, the yield of M_s is not a quadratic function of the flash intensity. The nonzero relative weight of M_s at low light intensities results in a linear dependence of the saturation curve of M_s in this intensity range. At higher light intensities the saturation makes it difficult to see a quadratic dependence.

Note that the rather complicated dependence of the relative weights of M_s versus the actinic light density previously reported by us (29) and shown in Fig. 5*a* very well fit the theoretical curve (Fig. 5*a*, *solid line*) obtained by the above discussed extension of the description of the saturation to the case of the cooperativity controlled M_s component.

In our measuring conditions (probably due to the solvent composition, the importance of which had been mentioned in Ref. 12) the yield of the M intermediate(s) (the peak amplitude at 412 nm) does not decrease with increasing temperature, as has been recently published by Fukuda and Kouyama (49). We were also not able to detect the increase in the relative weight of M_s with increasing temperature at low exciting flash intensities. The relative weight of M_s versus p is also independent of the temperature in the range of 10–50°C (data not shown). However, a possible transformation (or the presence) of a certain part of the sample into N or into another state (including a possible heterogeneity of BR in the nonexcited ground state) (49) could provide only a light intensity-independent contribution of M_s, and it could account for only the ~15% relative weight of M_s appearing at very weak flash intensities. The temperature independence of the yield of M_s is additional indirect evidence for cooperativity, since in the case of the presence of a heterogeneous ground-state population one could expect that the conformers would start to merge into one by increasing the temperature.

In conclusion we would like to emphasize that the actinic light energy density (and cooperativity) plays a crucial role in the photochemistry of BR. The dependence of the BR photocycle mechanism on the density of the exciting light is significant under a wide range of conditions (pH 7–9.5, low and high salt, −16°C to 50°C, etc.). However, it is easier to observe at high pH, where the lifetimes are more separated.

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