Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6358–6361, September 1988
Biophysics

Independent photocycles of the spectrally distinct forms of bacteriorhodopsin

(purple membrane/M intermediate/R intermediate/difference spectra/*Halobacterium halobium*)

Zs. Dancsházy, R. Govindjee, and T. G. Ebrey

Department of Physiology and Biophysics, University of Illinois, Urbana, IL 61801

Communicated by M. A. El-Sayed, May 23, 1988

ABSTRACT **Time-resolved, flash-induced difference absorbance spectra (300–700 nm) at pH 10.5 and 5°C for the bacteriorhodopsin photocycle fast and slow decaying forms of the M intermediate (M^f and M^s, respectively) and R intermediate are reported. The main distinguishing features are as follows: For M^f, ΔA_{max} = 412 nm, a shoulder at 436 nm, no absorbance change at 350 nm; ΔA_{min} = 565 nm; $\Delta A_{412}/\Delta A_{565}$ = 0.85. For M^s, ΔA_{max} = 412 nm, a shoulder at 386 nm; ΔA_{min} = 575 nm; $\Delta A_{412}/\Delta A_{575}$ = 0.6. For R, ΔA_{max} = 336 and 350 nm (double peak), smaller peaks at 386 and 412 nm; ΔA_{min} = 585 nm; $\Delta A_{350}/\Delta A_{585}$ = 0.2. The different difference spectra for M^f and M^s provide direct evidence that these species, initially identified by their kinetics, are physically distinct. With fast transient absorption spectroscopy, it was shown that R may form very fast, perhaps faster than the L intermediate decays. On the basis of the different bleaching peaks for M^f and M^s, we propose that M^f and M^s are in independent photocycles formed from slightly different forms of bacteriorhodopsin. R may also be in a different photocycle. The different forms of bacteriorhodopsin are probably in dynamic equilibrium with their ratios, controlled by pH and temperature.**

Upon light absorption, bacteriorhodopsin (BR) undergoes a photochemical cycle through several distinct intermediate states, termed K, L, M, O, and R (for review, see ref. 1 and below). The total number of the intermediates, their pathways, and their interrelationships are still under debate (e.g., refs. 2 and 3). The later intermediates of the photocycle, especially the one(s) absorbing at ≈410 nm (M), have been a particular focus of study because they are believed to play a key role in the proton-pumping function of BR. The initial models of the photocycle were linear and unbranched (1), but later kinetic studies showed these models were too simple because the decay of many of the intermediates was multiexponential (e.g., ref. 4). Some workers suggested that these multiexponential kinetics represent physically distinct species in sequential (5) or parallel (e.g., refs. 6 and 7) branched pathways. Others have stressed that such multiexponential processes can arise from a single species in equilibrium with other intermediates (8). El-Sayed and coworkers (9) have proposed that the biphasic kinetics seen in the rise of M are due to two chromophore environments provided by the apoprotein. In essence they have suggested that before photoexcitation BR exists in two forms, determined by the ionization state of a group on the apoprotein with a pK value of ≈9.6.

In this paper, we present strong evidence that the two forms of M, the fast and the slow decaying forms (M^f and M^s, respectively), that have been resolved kinetically at high pH represent two distinct physical species, since they have different extinction coefficients. Moreover, we have found that the BRs to which the different forms of M decay back have slightly different absorption spectra, suggesting that these forms of M arise from two spectrally different BRs. Finally, we propose that R, the very slowly decaying photocycle intermediate (10–12), may be in a different photocycle than either of those containing the M forms.

MATERIALS AND METHODS

Purple membranes were isolated from *Halobacterium halobium*, S9 strain, and suspended in 30 mM piperazine/30 mM glycylglycine, pH 10.5. All samples contained 40% (vol/vol) glycerol except those used for the flash-induced absorbance changes in Fig. 4. All measurements were carried out on light-adapted BR.

Two spectrophotometers were used to obtain the light-induced difference spectra. For the faster measurements, the data were taken and analyzed as described (10, 11). For the slower measurements a Hewlett–Packard model 8452A diode array spectrophotometer was used to take complete light-induced difference spectra from 300 to 700 nm with 2-nm spectral resolution. (The absorption spectrum of the light-adapted sample was used as the baseline.) The actinic source was a commercial photoflash with Corning long-pass filter (CS 3-67, wavelength >550 nm). The half-duration time was 150 μs. Since the measuring beam of the diode array spectrophotometer was relatively strong white light, special care was taken to check and avoid a possible actinic effect of the measuring beam. Sample OD_{570} was between 1.3 and 1.5.

RESULTS

Separation of the Difference Spectra of Intermediates M^f, M^s, and R. From the time-dependent series of difference spectra, we can determine the difference spectra of the late intermediates involved in the BR recovery at high pH (M^f, M^s, and R). We have chosen conditions for which the differences in the lifetimes and yields of these three components are maximal. We assume that the intermediates before M can be neglected because of their much shorter lifetimes and that no significant amount of the late intermediate O is formed because of the low temperatures and high pH values (refs. 5, 6, and 10 and unpublished data).

Fig. 1 is a typical data set (pH 10.5, 5°C) from the diode array spectrophotometer consisting of a series of 31 time-resolved difference spectra induced by a single actinic flash showing the decay of M and the reformation of BR. For clarity only six traces (traces 1, 2, 4, 8, 16, and 31) are shown. The time-dependent changes in the difference spectra are not uniform over the entire spectrum. Between traces 1 and 4 >60% of ΔA_{412} has decayed, while the ΔA_{330} and ΔA_{570} have decreased only ≈35% and ≈44%, respectively. Also a slight

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BR, bacteriorhodopsin; M^f and M^s, fast and slow decaying forms of the M intermediate.

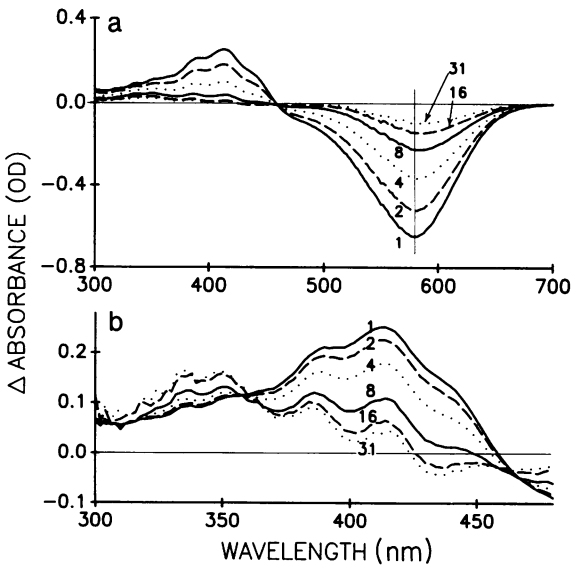

FIG. 1. Time dependence of the flash-induced spectral changes of BR at pH 10.5 and 5°C. For clarity only 6 traces are plotted of the 31 traces recorded at this sampling rate of 1 s^{-1} (data integration time, 0.5 s) starting ≈0.5 s after the actinic flash. (*a*) Full spectra as recorded. The vertical line is drawn at the minimum of difference spectrum 1 (≈575 nm) to show the shift of the minimum with time. (*b*) Spectra from *A* were normalized at their bleaching peaks (570–585 nm) and replotted in the 300- to 500-nm wavelength range.

red shift can be seen in the bleaching minimum at ≈570 nm in the spectra taken at later times (Fig. 1*a*).

In Fig. 1*b* the difference spectra from Fig. 1*a* are plotted from 300 to 480 nm after their normalization at 570–580 nm. The last spectrum has a residual ΔA of only ≈10% of the initial amplitude. The striking decrease in the $\Delta A_{412}/\Delta A_{570}$ ratio between the spectra taken at early and later times is accompanied by changes in the fine structure at ≈412 nm. Besides the main absorbance peak at 410–412 nm, peaks and shoulders are clearly resolved at 336, 350, 386, and 436 nm, decaying with different rates. Similar data as in Fig. 1 were seen at pH 7.2 and 9, from −15°C to 0°C (data not shown). The spectra shown in Fig. 1 indicate that a mixture of two or more spectrally and kinetically different BR photocycle intermediates is decaying back to BR under these conditions. Earlier studies suggested that these are the two forms of the M intermediate and the R intermediate (10, 11).

Fig. 2 shows semilogarithmic plots of the time-dependent absorbance changes at three wavelengths, 350, 412, and 580 nm (taken from the data set shown in Fig. 1 but also with a 2-s sampling interval). In each case the decay/recovery rates are multiexponential. The slowest component seen in Fig. 2*b* has a $t_{1/2}$ of 37 s at all three wavelengths. Moreover, difference spectra corresponding to the last 10–15 points plotted in Fig. 2*b*, when normalized at 580 nm, were superimposable at all wavelengths, showing that only one component from the initial mixture is left to decay at these late times. This implies that these late difference spectra represent a single intermediate. These difference spectra (Fig. 1*b*, trace 31, and Fig. 3) have the following characteristics: major absorbance maxima at 336 and 350 nm, minor maxima at 386 and 412 nm, and a bleaching minimum at 585 nm. We assign this difference spectrum to the R intermediate because of its long decay time and its striking similarity with the R spectrum reported earlier (11). Because of the likelihood that the absorption maximum of R is close to that of its parent pigment (11, 12), the difference spectrum does not provide a good estimate for the maximum of either the parent pigment or the R intermediate.

We can determine the contribution of R to the spectra plotted at early times in Fig. 1*a* by extrapolating through the last data points (Fig. 2*a*) with the slope obtained from Fig. 2*b* ($t_{1/2}$, 37 s). After subtracting the contribution of R from the ΔA_{412}, the resulting curve was nonlinear so we further resolved the decay into two components M^f and M^s. The two half-decay times obtained from the peeling of the ΔA_{412} trace after the subtraction of R were 1 and 3 s, respectively. These half-lives were confirmed in separate experiments with faster time resolution.

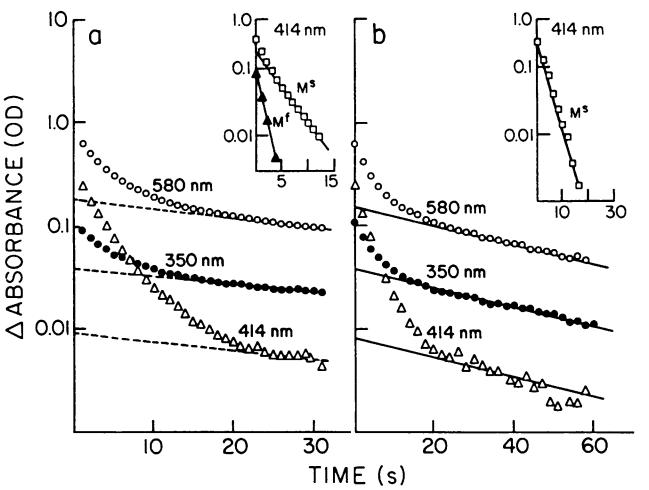

FIG. 2. Semilogarithmic plots of the time-dependent absorbance changes at 350, 412, and 580 nm, taken from 62 difference spectra recorded as in Fig. 1*a*. (*a*) Sampling rate of 1 s^{-1} (data integration time, 0.5 s). (*b*) Sampling rate of 0.5 s^{-1} (data integration time, 1 s). The dotted lines in *a* represent the contribution of the slowest component ($t_{1/2}$, 37 s) extrapolated from *b* (see text). (*Insets*) The decays assigned to M^s ($t_{1/2}$, 3 s) and M^f ($t_{1/2}$, 1 s) were obtained after subtraction of the slower component(s) (R or M^s + R, respectively) from the ΔA_{412} plots. The initial amplitude of M^f could not be determined because of the 0.5-s delay before data acquisition.

The difference spectrum of M^s was obtained by taking trace 5 from the data set of Fig. 1*a* at ≈6 s after the flash, when M^f had decayed completely so that only M^s and R were present, and subtracting the contribution of R from it. The difference spectrum of M^s thus obtained (Fig. 3) has the following features: ΔA_{max} = 412 nm, shoulders at 386 and 436 nm, and ΔA_{min} = 575 nm, $\Delta A_{412}/\Delta A_{575}$ = 0.6 ± 0.05.

The difference spectrum of M^f was obtained by subtracting the appropriate amount, as indicated above, of M^s and R from

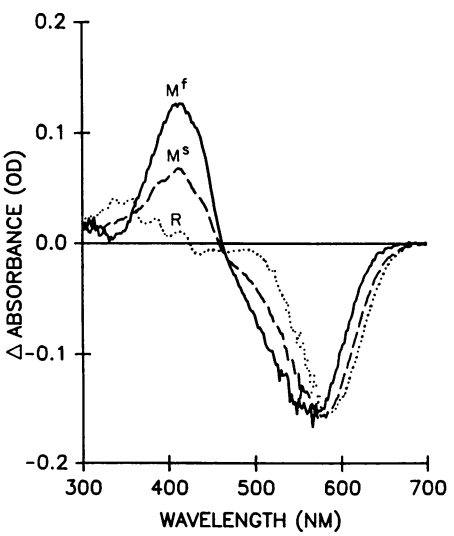

FIG. 3. Flash-induced difference absorbance spectra at 5°C and pH 10.5. The spectra are normalized at ≈570 nm. Note that not only do the different intermediates have different relative absorbances in the blue but also that the bleaching minima are different.

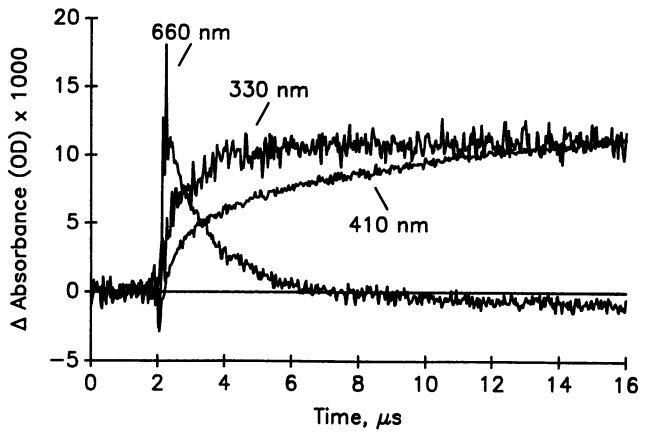

FIG. 4. Flash-induced absorbance changes on a fast time scale at 660 nm (a wavelength chosen to monitor the formation and decay of the K intermediate), 330 nm (R), and 410 nm (the M intermediates) at 20°C and pH 9.0.

trace 1 of the data taken with a 0.5-s sampling rate. The difference spectrum of M^f (Fig. 3) has the following features: ΔA_{max} = 412 nm, ΔA_{min} = 565 nm, shoulder at 436 nm, no absorbance at 350 nm, and $\Delta A_{412}/\Delta A_{565} = 0.85 \pm 0.05$. This spectrum of M^f is very close to the spectrum obtained by subtraction of the first two difference spectra obtained at −15°C at pH values from 7.2 to 10.5 (data not shown), a procedure that should isolate the most rapidly decaying intermediate. Two important distinguishing features between the difference absorption spectra of M^f and M^s are the positions of the bleaching peak (565 nm vs. 575 nm) and the ratio of ΔA_{412} with respect to the bleaching peak (0.85 vs. 0.6).

The Origin of R. To check the relationship between the M intermediates and the R intermediate, flash-induced kinetic absorbance measurements were made at selected wavelengths (330, 410, and 660 nm). Under our conditions (pH 9–10 and 5–20°C) the increase of M is biphasic. The absorbance increase at 330 nm is faster than the increase at 410 nm (due to the M intermediate) and is similar to the initial decay at 660 nm, which is due to the K intermediate (Fig. 4). The decay of R (ΔA_{330}) is much slower than the decays of M^f and M^s (10, 11). There are three possible explanations for the origin of the absorbance increase at 330 nm. The first (and simplest) is that most of this change is due to the formation of the R intermediate, since R absorbs very strongly at this wavelength. If so, this would suggest that R is not formed from either M^f or M^s and thus is in a different photocycle, as shown in Fig. 5. A second explanation is that the absorbance increase at 330 nm is a "cis" peak associated with all of the intermediates that have a 13-cis conformation; this is probably incorrect because although it is widely acknowledged that K has a 13-cis conformation, the absorption increase at 330 nm is still not complete at a time when all of K has been formed (Fig. 4). A third possibility is that all of the intermediates after K, such as L and M as well as R, absorb at 330 nm; in this case no conclusion can be made about the origin of R.

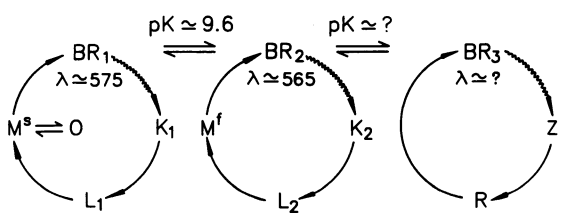

FIG. 5. Schematic diagram of the photocycles of the different forms of BR. BR_1 is the form that predominates near neutral pH, and BR_2 becomes important as the pH increases. At alkaline pH values, BR_3 also becomes increasingly important. Z, primary photoproduct of BR_3, perhaps the precursor of R.

DISCUSSION

El-Sayed and coworkers (9) showed that the biphasic decay of the L intermediate from pH 7 to pH 11 behaved as if it originated from two forms of BR that were in a pH-dependent equilibrium with a pK value of ≈9.6. This implies one should be able to detect a difference in the two pigments. The simplest interpretation of our difference spectra (Fig. 3) is that there are at least two and perhaps three distinct forms of BR from pH 7 to pH 11; each probably has a different absorption spectrum and gives rise to spectrally distinct intermediates, implying that there are two and perhaps three different photocycles (Fig. 5).

The form of BR that predominates around neutral pH (10, 11) we term BR_1; BR_1 gives rise to M^s and probably the O intermediate. BR_2, which predominates as the pH increases (11), gives rise to the M^f intermediate. It absorbs at slightly shorter wavelengths than BR_1. From the difference spectra (Fig. 3), M^s has a lower extinction coefficient than M^f or BR_2 has a lower extinction coefficient than BR_1 or both. The more hypothetical BR_3, which may be the parent of R, is also important at high pH values (11). For completeness we should mention the long wavelength form of BR (λ_{max} = 603 nm) that is formed at very low pH values (the blue membrane) and does not have a M-type intermediate (e.g., ref. 13).

There is some evidence for two forms of BR with slightly different absorption spectra interconverting with a pK value of ≈9.7. BR in which the native retinal had been replaced by 3,4-dihydroretinal had a very clearly discernible pH-dependent absorption spectrum with a pK value of 9.7, with the longer-wavelength, higher-extinction form predominating at neutral pH (14). Others have also found a similar but less-pronounced phenomenon in native BR (15, 16). Two spectrally distinct forms of light-adapted BR have been observed at 77 K (17). Thus, there is evidence from absorption and difference spectra for multiple pH-dependent forms of BR.

There is also some evidence indicating distinct forms of M, but it is mostly either indirect (6, 18, 19) or extremely brief (20). Most other spectroscopic attempts to show that the two forms of M are spectrally or physically distinct have not been successful (2, 7, 21), probably because of lack of resolution or inappropriate conditions. However, during the preparation of this manuscript it was reported (22) that M^f and M^s have different Fourier transform infrared spectra and that they are in independent photocycles. That there are different photocycles for different BRs, varying with pH (and perhaps temperature and membrane potential) (23), immediately raises a question about which form of BR (and M) was present in many previous experiments in which its properties were studied.

We are grateful to Drs. Pál Ormos and Lajos Keszthelyi for many useful discussions and to Burr Nelson and S. Y. Liu for invaluable assistance. This work is supported in part by grants from the National Science Foundation (PCM DMB 1 85-15339) and the Department of Energy (ER12087).

1. Stoeckenius, W., Lozier, R. & Bogomolni, R. (1979) *Biochim. Biophys. Acta* **505**, 215–218.
2. Maurer, R., Vogel, J. & Schneider, S. (1987) *Photochem. Photobiol.* **46**, 247–253.
3. Xie, A. H., Nagle, J. F. & Lozier, R. (1987) *Biophys. J.* **51**, 627–635.
4. Groma, G. I. & Dancsházy, Zs. (1986) *Biophys. J.* **50**, 357–366.
5. Lozier, R. H., Niederberger, W., Ottolenghi, M., Sivorinovsky, G. & Stoeckenius, W. (1978) in *Energetics and*

Structure of Halophilic Microorganisms, eds. Caplan, S. R. & Ginzburg, M. (Elsevier/North Holland, Amsterdam), pp. 123–139.
6. Li, Q., Govindjee, R. & Ebrey, T. G. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7079–7082.
7. Groma, G. I., Helgerson, S. L., Wolber, P. K., Beece, D., Dancsházy, Zs., Keszthelyi, L. & Stoeckenius, W. (1984) *Biophys. J.* **45**, 985–992.
8. Parodi, L. A., Lozier, R. H., Bhattacharjee, S. & Nagle, J. F. (1984) *Photochem. Photobiol.* **40**, 501–512.
9. Hanamoto, J. H., Dupuis, P. & El-Sayed, M. A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7083–7087.
10. Dancsházy, Zs., Govindjee, R., Nelson, B. & Ebrey, T. G. (1986) *FEBS Lett.* **209**, 44–48.
11. Dancsházy, Zs., Govindjee, R. & Ebrey, T. G. (1987) in *Biophysical Studies of Retinal Proteins*, eds. Ebrey, T., Frauenfelder, H., Honig, B. & Nakanishi, K. (Univ. of Illinois, Urbana), pp. 167–173.
12. Drachev, L. A., Kaulen, A. D., Skulachev, V. P. & Zorina, V. V. (1986) *FEBS Lett.* **209**, 316–320.
13. Chang, C.-H., Chen, J.-G., Govindjee, R. & Ebrey, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 396–400.
14. Tokunaga, F. & Ebrey, T. (1978) *Biochemistry* **17**, 1915–1922.
15. Muccio, D. D. & Cassim, J. Y. (1979) *J. Mol. Biol.* **135**, 595–609.
16. Palmer, P. & Sherman, W. (1985) *Photochem. Photobiol.* **42**, 541–547.
17. Balashov, S. P., Karneeva, N. V., Imasheva, E. S. & Litvin, F. F. (1986) *Biofizika* **31**, 981–984.
18. Hess, B. & Kuschmitz, D. (1977) *FEBS Lett.* **74**, 20–24.
19. Ohno, K., Govindjee, R. & Ebrey, T. (1983) *Biophys. J.* **43**, 251–254.
20. Mathew, M. K., Helgerson, S. L., Bivin, D. & Stoeckenius, W. (1985) *Biophys. J.* **47**, 323 (abstr.).
21. Deng, H., Pande, C., Callender, R. H. & Ebrey, T. G. (1985) *Photochem. Photobiol.* **41**, 467–470.
22. Ormos, P., Braunstein, D., Hong, M., Lin, S. & Vittitow, J. (1988) *Biophys. J.* **53**, 380 (abstr.).
23. Westerhoff, M. & Dancsházy, Zs. (1984) *Trends Biochem. Sci.* **9**, 112–116.