# PHOTOLYSIS OF BACTERIAL RHODOPSIN

M. CHU KUNG, DON DEVAULT, BENNO HESS, *and* DIETER OESTERHELT

*From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19174, the Max-Planck-Institut für Ernährungsphysiologie, Dortmund, West Germany, and the Institut für Biochemie der Universität Würzburg, Würzburg, West Germany*

We have investigated the kinetics of the formation of the 412 nm product of the photolysis of suspensions of purple membrane from *Halobacterium halobium*. This was an extension of the kinetic studies of Oesterhelt and Hess (1) who measured the recovery time in a suspension saturated with diethyl ether to slow the reaction. In the present work we used faster spectrophotometric techniques (2) and did not use ether. In most of the measurements we did slow the reactions by cooling to −25°C, using 25% ethylene glycol in the "basal salt" solution (1) to prevent freezing. We found and measured several intermediates, confirming in general the results of similar independent studies by Lozier et al. (3) and by Dencher and Wilms (4). In a parallel project, Chance et al. (5) studied the kinetics of the accompanying proton movements. A preliminary report was made to the 1975 Biophysical Society meeting (6).

The suspensions of purple membrane were prepared in Germany from the mutant *H. halobium* NRL $R_1M_1$ and isolated as described in reference 7. Activation of the sample was usually by a 20 ns pulse of light of 539 nm wavelength, shifted by stimulated Raman effect (8) in hydrogen at 75 atm from the 694 nm light emitted by a Q-switched ruby laser. This arrangement also produced about 1/10 as much light at 440 nm which was sometimes removed with a yellow filter. In one series of experiments, activation was by 1 μs. pulses of 585 nm wavelength light from a Rhodamine 6-G dye laser. At the sample, the pulses from the ruby-$H_2$ laser had an intensity in the neighborhood of 2 $mJ \cdot cm^{-2}$ or 9 $nE \cdot cm^{-2}$, while the irradiation from the dye laser varied from about 10 to 2 $mJ \cdot cm^{-2}$ (50–10 $nE \cdot cm^{-2}$). The cuvette area was 15 mm × 15 mm with a light path of 2 mm. The concentration of bacteriorhodopsin was usually about 80–100 μM (16–20 $nmol \cdot cm^{-2}$ area density) measured by absorption at 568 nm with extinction coefficient (1) taken as 63 $mM^{-1} \cdot cm^{-1}$. The sample was normally in a "light-adapted" condition (3, 9), that is it would have been exposed to light within the 15 min preceding a measurement, but not within 2 min.

Fig. 1 summarizes measurements of the rate of formation of the 412 nm compound

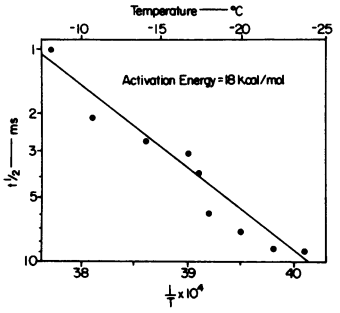


FIGURE 1

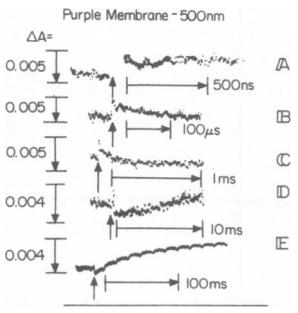


FIGURE 2

FIGURE 1 Arrhenius plot of the temperature dependence of the rate of formation of the 412 nm ($R_4$) compound.

FIGURE 2 Time course of optical density changes at 500 nm wavelength. Increase of optical density is downward. Upward-pointing arrows indicate time of laser flash. A and E are single shots. B, C, and D are the results of averaging 7, 5, and 7 shots, respectively, with a Varian Associates C-1024 time averaging computer accepting data from a Biomation 610 transient recorder. Concentration of samples, about 100 $\mu$M (20 nmol·cm$^{-2}$). Light path, 2 mm. Temperature −25°C.

(called "$R_4$" in this paper) as a function of temperature in an Arrhenius plot. Previous measurements (1) were light-limited rates. Those in Fig. 1 are dark reaction rates following absorption of the short light flash. The activation energy is 18 kcal/mol.

Oscilloscope traces illustrating the time course of the absorption changes at 500 nm from 100 ns to 100 ms following the flash are shown in Fig. 2. At this wavelength one sees first an optical density decrease (upward in Fig. 2) in less than 200 ns, trace A. This ends in an intermediate we call $R_2$. The existence of $R_1$, a supposed transient occurring before $R_2$ is suspected but not proven. Then with a half-time of 83 $\mu$s (average of six determinations) the absorbency increases to a new value in trace C characteristic of another intermediate, $R_3$. Then with a half-time usually 12 ms at −25°C, the absorbency decreases as compound $R_4$ is formed. In trace E, an early measurement, the half-time appears longer because too much measuring light produced an altered steady state. The rest of the data in Fig. 2 and those in Figs. 3 and 4 were taken with measuring light too dim to affect the results.

Similar measurements at different wavelengths produce the difference spectra shown in Fig. 3. The results of experiments done with different laser intensities were normalized to that prevailing in the excitations by the ruby-$H_2$ laser in the May series. The curves of Fig. 3, added to the absolute absorption spectrum of an amount of the starting compound, $R_0$, assumed to be equivalent to the amount photolyzed give the absolute spectra for the intermediates shown in Fig. 4. The amount of $R_0$ chosen to match the curves of Fig. 3 was 0.23 nmol·cm$^{-2}$, indicating this was the amount of photolysis per flash in the experiments of Fig. 3. Thus the photolysis was about 1.3% and quantum efficiency in the neighborhood of 3%. At room temperature the quantum efficiency was measured (1) as 79%.

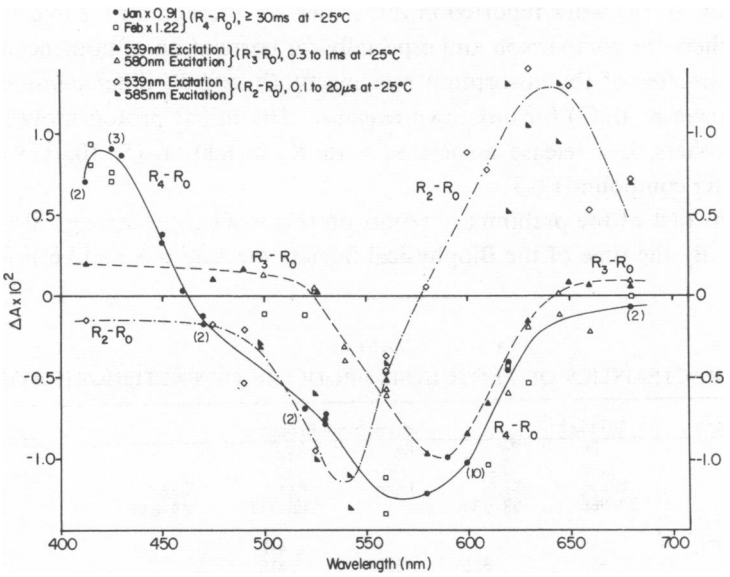


FIGURE 3 Difference spectra obtained at −25°C from data similar to that in Fig. 2. Six series of measurments are represented: •, data taken January 1975 normalized to the May series by multiplying by 0.91; □, data of February 1975 normalized by a factor of 1.22; △, data of April 1975 appropriate to time range 0.3–1 ms, excitation by 585 nm dye laser; ▲, data of May 1975 and April 30, 1975, appropriate to same time range, excitation by 539 nm ruby-$H_2$ laser; ▸, data of April appropriate to time range 0.1–20 μs, dye laser excitation; ◇, data of May 1975 and April 30, 1975, in 0.1–20 μs time range, ruby-$H_2$ laser excitation. The dye laser data was normalized to the May levels by factors which varied from day to day. Numbers in parentheses beside certain points indicate the number of single shots averaged into the given point. In addition most of the April and May points are averages of 4–10 shots made with the time-averaging computer.

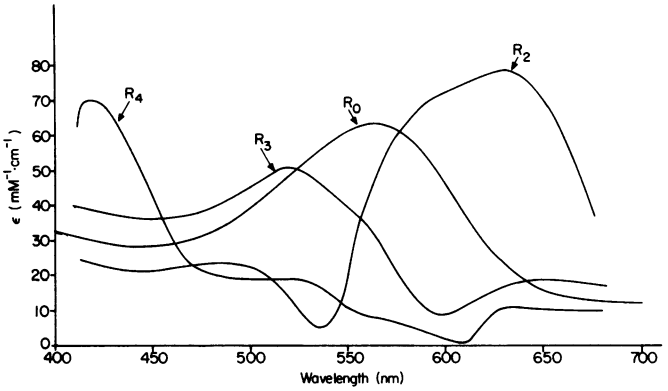


FIGURE 4 Absolute absorption spectra of the original compound, $R_0$, and successive products of photolysis, $R_2$ to $R_4$, derived from the curves of Fig. 3 by adding each to an $R_0$ spectrum corresponding to 0.23 nmol·cm$^{-2}$ of bacteriorhodopsin.

The results of the work reported in this paper are presented in Table I along with those of others for comparison and especially for correlation of nomenclature. Note that our estimates of the absorption maxima of $R_2$ and $R_3$ differ considerably from those of Lozier et al. (3) for unknown reasons. The initial proton movement, determined by others, is a release associated with $R_4$ formation (5, 10, 11) or possibly that of a later compound (3–5).

In the abstract of the preliminary report on this work (6) a strong role for $R_1$ was suggested. By the time of the Biophysical Society meeting this had been found to be

TABLE I

CHARACTERISTICS OF PHOTOLYSIS PRODUCTS OF BACTERIORHODOPSIN

| Compound Designation | Initial $R_0$ | Successive Products $R_2$ | $R_3$ | $R_4$ | | References* |
|---|---|---|---|---|---|---|
| | LA bR570 BR-565 | LA K590 BR-630 | LA L550 | LA M412 BR-411 | LA O640 BR-646 | K<br>L<br>D |
| Abs. max. (nm) | 568 | | | 412 | | O |
| | 563 | 631 | 520 | 418 | | K |
| | 570 | 590 | 550 | 412 | 640 | L |
| $\epsilon$ ($mM^{-1}\cdot cm^{-1}$) | 63 | 78 | 51 | 70 | | O,K |
| | 63 | 53 | 46 | 41 | 50 | L |
| Diff. spectra from $R_0$ max (nm) | | 640 | 420?,670? | 420 | | K |
| | | 637 | 500?,662? | 412 | | L |
| | | 630 | | 411 | 646 | D |
| Min. (nm) | | 539 | 587 | 569 | | K |
| | | 550 | 589 | 579 | | L† |
| | | 535 | | 572 | | D |
| Isosbestic (nm) | | 578 | 525,643 | 462 | | K,O |
| | | 590 | 520,650 | 464 | 600 | L |
| | | 596 | | 458 | 623 | D |
| Formation half-time | | ≤0.1μs | 83μs | 11.5ms | | K,-25°C |
| | | | | 5ms | | D,-25° |
| | | | | 1ms | | K,-8° |
| | | | | 0.32ms | | D,0° |
| | 100ms | < 1μs | 4.5μs | 0.3ms | | L,1° |
| | 22ms | | | | 9ms | D,10° |
| | | | 2μs | | | L,25° |
| | 2.4ms | | | | 2ms | D,30° |
| | 40 s | | | | | D, 3°,ether |
| | 110 s | | | | | O,5°, ether |
| $E_A$, §(kcal·mol$^{-1}$) | 11.4 | | | | | O, ether |
| | | | 15.2 | 18 | | K,L ‖ |
| | | | 5.5 | | | L ** |
| | 19 | | | 14.1 | 13 | D |
| Proton movement on formation | Uptake | | | Release | | O,C,Lewis |
| | Uptake | | | | Release | L,C |
| | Release | | | | Uptake | D |

*K, Kung et al., this paper; L, Lozier et al. (3); D, Dencher and Wilms (4); O, Oesterhelt and Hess (1); C, Chance et al. (5); Lewis, Lewis et al. (11).

‡Reconstructed from Fig. 5 of ref. 3 by summing appropriate curves.

§Energy of activation calculated from values given above in this table if not given in original papers.

‖ Value for $R_3$ is from rates at 1° and −25° above. Value for $R_4$ is from Fig. 1.

**From rates at 1° and 25° given above.

mostly laser-induced artifact of a type that is not detected by observing the flash with measuring light turned off. The test for it is to substitute an inert sample or to remove the sample completely. The transient, $\Delta A$-mimicking artifact then depends on the strength of measuring light for both direction and amplitude. Such artifacts were carefully avoided in the work presented here. The existence of $R_1$ is left questionable. Curve A of Fig. 2 is not incompatible with a rise half-time for $R_2$ or $R_1 + R_2$ of around 200 ns if the first 200 ns after the laser flash is ignored as laser artifact, but this would be speculation. It is possible that if $R_1$ exists it might be simply the triplet state of excited $R_0$.

We wish to thank Doctors Lozier, Bogomolni, and Stoeckenius and Doctors Dencher and Wilms for allowing us to see their manuscripts before publication.

This research was supported by U.S. Public Health Service grant GM-12202, National Science Foundation grant GB-27600, and the Max-Planck Gesellschaft.

*Received for publication 16 June 1975.*

## REFERENCES

1. OESTERHELT, D., and B. HESS. 1973. Reversible photolysis of the purple complex in the purple membrane of *Halobacterium halobium*. *Eur. J. Biochem.* **37**:316. HESS, and D. OESTERHELT. 1974. Reversible photolysis of the purple complex from *Halobacterium halobium* as a basis for the function of the purple membrane. *In* Dynamics of Energy-Transducing Membranes. L. Ernster, R. W. Estabrook, and E. C. Slater, editors. Elsevier Scientific Publishing Co., Amsterdam. 257.
2. DEVAULT, D. 1964. Photochemical activation apparatus with optical laser. Rapid Mixing and Sampling Techniques in Biochemistry. B. Chance, Q. Gibson, R. Eisenhardt, and K. Lonberg-holm, editors. Academic Press, Inc., New York. 165.
3. LOZIER, R. H., R. A. BOGOMOLNI, and W. STOECKENIUS. 1975. Bacteriorhodopsin: a light driven proton in pump in *Halobacterium halobium*. *Biophys. J.* **15**:955.
4. DENCHER, N., and M. WILMS. 1975. Flash photometric experiments on the photochemical cycle of bacteriorhodopsin. Biophysics of Structure and Mechanism. **1.** In press.
5. CHANCE, B., M. PORTE, B. HESS, and D. OESTERHELT. 1975. Low temperature kinetics of $H^+$ changes of bacterial rhodopsin. *Biophys. J.* **15**:913.
6. DEVAULT, D., M. CHU KUNG, B. HESS, and D. OESTERHELT. 1975. Photolysis of bacterial rhodopsin. *Biophys. J.* **15**(2,Pt.2):67*a*. (Abstr.).
7. OESTERHELT, D. 1973. Reversible dissociation of the purple complex in bacteriorhodopsin and identification of 13-cis and all-trans-retinal as its chromophores. *Eur. J. Biochem.* **40**:453.
8. MINCK, R. W., R. W. TERHUNE, and W. G. RADO. 1963. Laser-stimulated Raman effect and resonant four-photon interactions in gases $H_2$, $D_2$ and $CH_4$. *Appl. Phys. Let.* **3**:181.
9. STOECKENIUS, W., and R. H. LOZIER. 1974. Light energy conversion in *Halobacterium halobium*. *J. Supramol. Struct.* **2**:769.
10. OESTERHELT, D., and W. STOECKENIUS. 1973. Functions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. U.S.A.* **70**:2853.
11. LEWIS, A., J. SPOONHOWER, R. A. BOGOMOLNI, R. H. LOZIER, and W. STOECKENIUS. 1974. Tunable laser resonance Raman spectroscopy of bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* **71**:4462.