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FLASH KINETIC STUDY OF THE LAST STEPS IN THE PHOTOINDUCED REACTION CYCLE OF BACTERIORHODOPSIN

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Summary

The reaction cycle of light adapted bacteriorhodopsin (BR) in aqueous purple membrane suspensions was studied by laser flash photolysis at different temperatures (2—49°C) and pH values (3—10). The activation energy for several reaction steps was determined at pH 7.6. The kinetics of O-bacteriorhodopsin (one of the last intermediates in the cycle) were analyzed in some detail and it was found that the simple consecutive reaction scheme M-BR → O-BR → BR may explain the kinetics of O-bacteriorhodopsin as measured at 680 nm. Since the pH change in neutral aqueous suspensions of purple membrane follows a similar kinetics as O-bacteriorhodopsin it is suggested that protons are released during the reaction M-BR → O-BR and taken up again during the reaction O-BR → BR.

Another long-lived intermediate, which absorbs to a greater extent than bacteriorhodopsin at 570 nm and less than bacteriorhodopsin at 420 nm, was identified with the strongly fluorescing species, pseudo- or P-bacteriorhodopsin. The decay of P-bacteriorhodopsin in bacteriorhodopsin had an activation energy of only approx. 1.2 kcal/mol, which suggests that the last step of the photocycle is a relaxation around a single bond.

At pH 9—10, the simple first-order kinetics of all the intermediates were changed into a kinetics consisting of two first-order decays. This change of kinetics was accompanied by a drastic decrease in the rotational diffusion relaxation time.

To explain the results obtained in this work and those of others, a model involving proton uptake and release by the Schiff base nitrogen combined with an isomerization reaction is finally proposed.

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Abbreviation: BR, bacteriorhodopsin.

Introduction

Bacteriorhodopsin in the purple membrane of *Halobacterium halobium* undergoes a photoinduced reaction cycle which has been studied by several groups with optical absorption spectroscopy using low-temperature and flash kinetic techniques [1—11]. At least four intermediates of the reaction cycle have been identified by their absorption spectra. There are, however, still some questions of considerable interest that deserve continued investigation. First, there is some controversy in the literature about the position and significance of O-bacteriorhodopsin [3—7], which is generally believed to be one of the last forms in the reaction cycle of light-adapted bacteriorhodopsin (BR). In this paper we have studied the kinetics of O-BR in some detail, especially has the temperature dependence been followed. Secondly, a new form of bacteriorhodopsin (P-BR), which is formed during illumination of light-adapted bacteriorhodopsin at 77 K, was recently identified [12]. P-BR should, according to luminescence measurements, have an optical absorption close to that of light-adapted bacteriorhodopsin and a lifetime of approx. 42 ms at 298 K (Gillbro, T., Kriebel, A. and Wild, U.P., unpublished). One purpose of this work was therefore to search for the optical absorption of P-BR at ambient temperatures, i.e. 0—50°C, and to see if P-BR might play an important rôle in the reaction cycle of light-adapted bacteriorhodopsin.

The activation energy for some of the reaction steps has been obtained. The number of kinetic measurements at $T > 35°C$ is, however, to small to allow a comparison with the data in refs. 6 and 7 to be made. We have also studied the effects on the reaction cycle caused by changing the pH of the purple membrane suspension. A marked effect on the kinetics and the polarization anisotropy was observed in alkaline solution (pH 9—10).

Throughout this paper we use the notations given by Lozier et al. [1] for the different intermediates of light-adapted bacteriorhodopsin.

Experimental

The purple membrane suspension was prepared according to the method given by Oesterhelt and Stoeckenius [13] and the samples used typically had an absorbance of 1.0 at 570 nm. The pH of the neutral aqueous solution used in most experiments reported in this paper was 7.6. For other pH values, buffered (Titrisol) or NaOH/HCl tritrated solutions were used. The temperature control was provided by an Oxford Instruments cryostat DN 704 cooled by liquid nitrogen.

The experimental arrangement used for the flash photolysis has been described before [14]. Briefly, the pulsed excitation source is a flashlamp-pumped dye laser with emission at 540 nm. The laser pulse width of approx. 2 μs sets the limit of the time resolution. The apparatus can be used for polarization measurements, since the laser excitation light is plane-polarized. The absorbance changes $A_{\parallel}(t)$ and $A_{\perp}(t)$ at time t after the flash for light polarized parallel and perpendicular to the polarization of the exciting flash were calculated from oscilloscope traces of the photomultiplier response or, alternatively, the accumulated signal from 16 flashes was stored in a Datalab

D102 A signal averager and then processed on a HP 9825A computer. Both the total absorbance change, $A_{\parallel}(t) + 2A_{\perp}(t)$, and the polarization anisotropy,

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)}$$

can be calculated. Bacteriorhodopsin was light adapted by the stabilized 100-W tungsten halide lamp used as a source of the measuring beam.

Results and Discussion

Before analyzing the kinetics of the build-up and decay of the different intermediates of light adapted bacteriorhodopsin we measured the polarization anisotropy, $r(t)$, in neutral (pH $\approx$7.6) suspensions of purple membrane.

When no significant time dependence of r could be detected over the time range under investigation, $A_{\parallel}(t)$ rather than $A_{\perp}(t)$ was measured in some of the work reported below.

The only time when the polarization anisotropy changed notably with time was in alkaline solution (pH 9—10) and more about this will be discussed in the end of this paper.

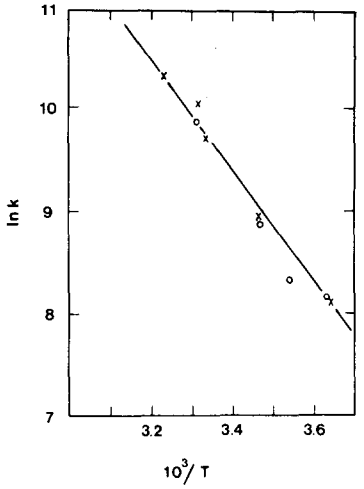
The photochemical cycle of light-adapted bacteriorhodopsin with special emphasis on O-BR

As mentioned in the introduction, one of the aims of this paper was to study the kinetics of O-BR, which is an intermediate of light-adapted bacteriorhodopsin absorbing most strongly at approx. 640 nm [1], in some detail. In order to do so, however, some information on the other intermediates in the cycle was needed. The fastest time resolution of our spectrometer is not sufficiently fast to study the K-form ($t_{1/2} \approx 2\ \mu s$) at ambient temperatures. The kinetics of L-BR which is thought to be the second intermediate of the cycle [1], were studied at 570 nm. The decay of L-BR can be fitted to a first order rate equation with the rate constant, k_L. Ln k_L is plotted against T^{-1} (K) in Fig. 1. The same study was made of the formation of M-BR at 430 nm which could also be fitted to a first order rate equation (rate constant k_M). Ln k_M is plotted against T^{-1} in Fig. 1. We have fitted the best straight line through the points in Fig. 1 that represents the decay of L-BR. The activation energy of the decay of L-BR can then be calculated to be 10.7 kcal/mol. The points representing the formation of M-BR are more scattered, probably due to the poorer experimental conditions at 430 nm (light scattering), but they are close to or on the straight line representing the decay of L-BR. It therefore seems very likely that the reaction

$$\text{L-BR} \xrightarrow{k_L} \text{M-BR}$$

is a first order reaction with an activation energy of 10.7 ± 0.5 kcal/mol in a neutral (pH $\approx$7.6) aqueous suspension of purple membrane.

The following steps in the photocycle are less well understood [1,3,5—7]. For instance, it is not clear whether M-BR or O-BR is the precursor of BR, or if O-BR is formed in parallel or in series to M-BR. The main reasons why this part of the photocycle is not clearly understood are, firstly, the different



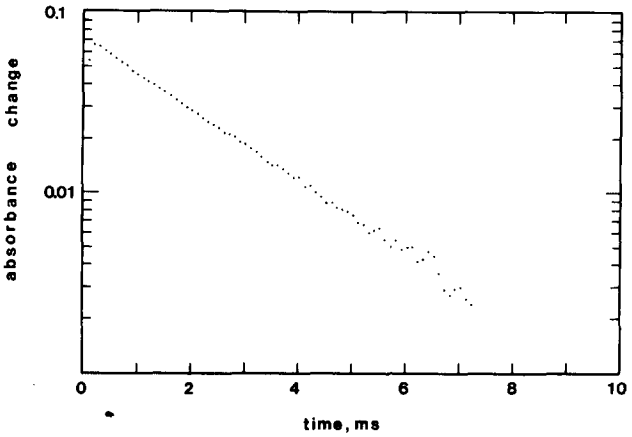


Fig. 1. Arrhenius plot of ln k (s^{-1}) as a function of $1/T$ (K) for the decay of L-BR (X) and formation of M-BR (○) in a purple membrane suspension at pH 7.6. Data from oscilloscope traces.

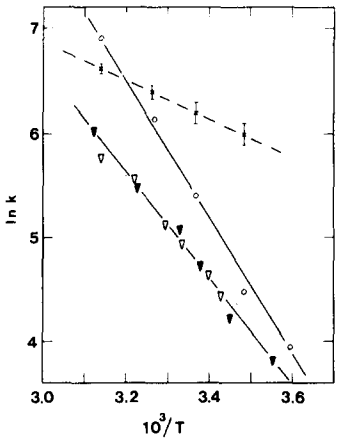
Fig. 2; Time course of the absorbance change ($\Delta A(t)$) at 420 nm in a neutral suspension of purple membrane at 33°C. Average of 16 flashes from dye laser.

kinetics for the decay of M-BR and build-up of BR (non-first order build-up of BR which is slower than the decay of M-BR) and, secondly, the temperature-dependent yield of O-BR (higher yield at 40°C than at 1°C) [1,5—7]. We did not detect the intermediate N-BR, which should have an absorption maximum around 520 nm [1].

In this section we wish to show that all the anomalies mentioned above might be qualitatively and quantitatively explained by assuming the following simple reaction path:

$$\text{M-BR} \overset{k_M}{\rightarrow} \text{O-BR} \overset{k_O}{\rightarrow} \text{BR} \qquad (1)$$

Starting with the decay of M-BR as measured at 420 nm it follows closely first-order kinetics (see Fig. 2). Ln k_M as a function of T^{-1} is plotted in Fig. 3. The straight line fitted to these points gives the reaction M-BR → O-BR an activation energy of about 13.1 kcal/mol. Since we measured just one rate constant at $T > 35°C$, the break in the Arrhenius plot at $T \approx 30°C$ as reported by Sherman et al. [6,7] could not be studied. As has been mentioned, it is more difficult to fit the kinetic data of the decay of O-BR and the build-up of BR with a single or several first-order reactions. As a crude first approximation we have obtained the kinetic data also presented in the form of an Arrhenius plot in Fig. 3. The data points so obtained fall close to the same straight line. This indicates that the precursor of BR probably is O-BR and not M-BR. However, there is still no satisfactory explanation for the relatively fast rise-time of the O-form (see Fig. 4) and the temperature-dependent yield of O-BR. In order to check the validity of the simple reaction sequence M-BR → O-BR → BR on the concentration of O-BR we just put the data of k_M and k_O in Fig. 3 for $T = 33.4°C$ in the equation for two first-order consecu-



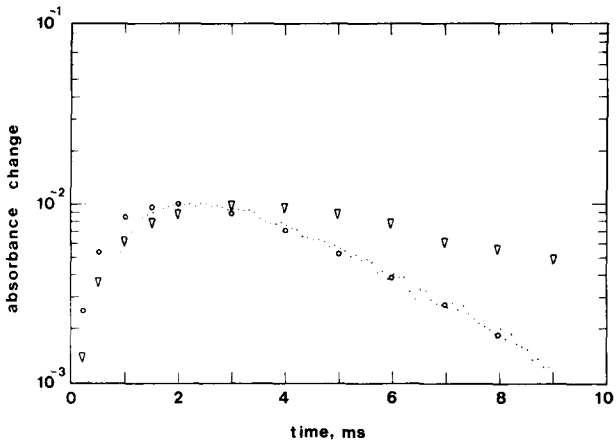


Fig. 3. Arrhenius plot of ln k (s^{-1}) as a function of $1/T$ (K) for (○) the decay of M-BR at 420 nm (mean value of 16 laser pulses) (▽) the decay of O-BR at 680 nm (from oscilloscope traces) (▼) the recovery of BR at 570 nm (from oscilloscope traces) and (X) the calculated values of k_O (see text).

Fig. 4. The absorbance change at 680 nm as a function of time at 33°C. The data points are mean values from 16 flashes (dotted line). The calculated transient of O-BR using Eqn. 2 are represented by: ▽ for $k_M = 441\ s^{-1}$ and $k_O = 208\ s^{-1}$; ○ for $k_M = 441\ s^{-1}$ and $k_O = 600\ s^{-1}$.

tive reactions:

$$[\text{O-BR}] = [\text{M-BR}]_0 \cdot (\exp(-k_M t) - \exp(-k_O t))k_M/(k_O - k_M) \quad (2)$$

Here $[\text{M-BR}]_0$ stands for the concentration of M-BR at time $t = 0$ assuming that all M-BR is formed immediately after the flash. This approximation is discussed further below.

As shown in Fig. 4 (open triangles) the decay of O-BR as calculated by Eqn. 2 (normalized to the experimental curve) fits poorly to the experimental data at 33.4°C, which seems to exclude this reaction sequence. However, there still remains the possibility that $k_O > k_M$, because in this case the decay of O-BR (and build-up of BR) will not be determined by k_O but mainly by k_M. We have thus tried different values for k_O in Eqn. 2 (keeping k_M constant) and in this way we could obtain a good fit to the experimental data in Fig. 4 (open circles). We carried out the same procedure at four different temperatures and obtained the data points as shown in Fig. 3. The errors in k_O calculated by this manual fitting procedure are of course rather large and are estimated in the figure. As can be seen in Fig. 4, the largest difference between the experimental and the calculated curve is found at shorter times. This difference will also be more pronounced as the temperature decreases. One explanation for this can qualitatively be found in the approximation made in the derivation of Eqn. 2 that M-BR is instantaneously formed from L-BR, i.e., all molecules in the cycle are in the M-form at $t = 0$. Fig. 1 gives, for instance, that $t_{1/2} = 57\ \mu s$ for the decay of L-BR at 294 K, which means that it will take 0.38 ms before 99% of L-BR has decayed into M-BR. At 312.5 K the corresponding contribution will be only 0.13 ms. This explains why the difference between experiment and theory increases with decreasing temperature. A second factor, which will distort the experimental signal from the real decay

of O-BR, is the small decrease in the absorption at 680 nm due to bleaching of BR just after the laser flash. The absorption signal from O-BR will thus be somewhat distorted during its rise, especially at lower temperatures when the concentration of O-BR is small and the recovery of BR is slow. This effect can be seen, for example, in Fig. 4, where the transient absorption crosses the baseline after approx. 0.3 ms.

As a further check of reaction 1 we have calculated from Eqn. 2 the time (t_{max}) it takes to reach maximum O-BR concentration using the data of k_M and k_O from Fig. 3 and have compared it to the maxima of the experimental curves at different temperatures (Table I). In the same table we have calculated the concentration of O-BR (I_{max}^{calc}) at t_{max} again using Eqn. 2 and compared it to the measured peak intensities (I_{max}^{exp}). The experimental and theoretical t_{max} are in good agreement considering that we have not corrected for the sources of error mentioned above. One consequently expects the experimental t_{max} to be longer than the theoretical, especially at lower temperatures. What is more important is that the theory also predicts the decrease of I_{max} as the temperature decreases [1,5—7]. This is because k_O/k_M gets larger as the temperature becomes lower as a consequence of the lower activation energy of 3.6 ± 1.0 kcal/mol (from Fig. 4) for the reaction O-BR → BR as compared to the reaction M-BR → O-BR.

From the discussion in this section it follows that the simple reaction sequence M-BR → O-BR → BR can explain very well the experimental results. The reason for its relative complexity is that $k_O > k_M$ at ambient temperatures.

Lozier et al. [15] observed that protons were released from the purple membrane into the aqueous phase with a half-time of approx. 0.8 ms at 21°C. The disappearance of the proton gradient had a half-time of 5.4 ms at the same temperature. However, the formation of M-BR, which is supposed to cause the proton release [15], occurs with a half-time of less than 0.1 ms at 21°C. This means that there is no direct connection between the pH change in the aqueous phase and the formation of M-BR. However, there is a pronounced similarity between the pH change reported by Lozier et al. and the kinetics of O-BR reported here. At 23.5°C for instance we measured a half-time for the build-up of O-BR of approx. 1.1 ms and using Eqn. 2 together with the rate

TABLE I

KINETIC DATA OF THE O-BR TRANSIENT

The content of this table is explained in the text.

T(K)	k_M^{exp} (s^{-1})	k_O^{calc} (s^{-1})	t_{max}^{calc} (s)	t_{max}^{exp} (s)	I_{max}^{calc}	I_{max}^{calc} *	I_{max}^{exp} *
287.0	88	450	4.51	10	0.132	0.31	—
296.7	222	500	2.92	4.5	0.232	0.54	0.5
305.9	462	600	1.89	2.4	0.321	0.74	0.7
318.7	1005	750	1.15	1.2	0.431	1.0	1.0

* These I_{max} values have been normalized at T = 318.7 K.

constants in Table I we get a calculated half-time of 0.7 ms for the O-BR formation at 23.5°C.

Furthermore the maximum of the pH change at 21°C is reached after approx. 3 ms [15] and according to our theoretical calculations (see Table I) the O-BR reaches its maximum concentration after 2.9 ms at 23.5°C. These results strongly suggest that protons are released into the aqueous phase during the reaction M-BR → O-BR and taken up again by the purple membrane during the reaction O-BR → BR. However, it is of course still possible that the release of protons into the medium is independent of the absorbance changes of the chromophore.

From Eqn. 2 it also follows that on calculating the extinction coefficient for O-BR (ϵ_0) one has to take into account that at the time (t_{max}) when the maximum absorbance change occurs, not all of the molecules going into the cycle are in the form of O-BR. One can, for instance, show that at 23.5°C only 23% of the molecules going into the cycle will be in the form of O-BR 2.9 ms after the laser pulse.

Evidence for the formation of P-BR also at ambient temperatures

In Fig. 5 is shown the transient signal at 570 nm from a neutral purple membrane suspension at 5°C after being excited by a laser flash at 540 nm. We note a recovery of the bleached absorption with a half-life of approx. 19 ms (Fig. 5a) followed by a signal which overshoots the baseline (Fig. 5b). This overshoot then slowly ($t_{1/2}$ = 84 ms) decays back to the baseline again. We have observed the same clear shooting signal at temperatures up to 25°C and at wavelengths from 490 to 610 nm.

Since this signal has not been reported in any previous work on flash photolysis of bacteriorhodopsin, we made several experiments in order to check whether it might be due to an artefact from our equipment or to some impurity. We analysed the kinetics of the decay and found it to be first order. Upon changing the temperature of the sample the rate constant for the decay also changed, which seems to eliminate all suspicions that the signal is an artefact due to the equipment (detector, electronics etc). The plot of ln k for the decay as a funciton of T^{-1} falls close to a straight line (Fig. 6). The activation energy was calculated to be approx. 1.2 kcal/mol, which is much smaller than for other known reactions of bacteriorhodopsin.

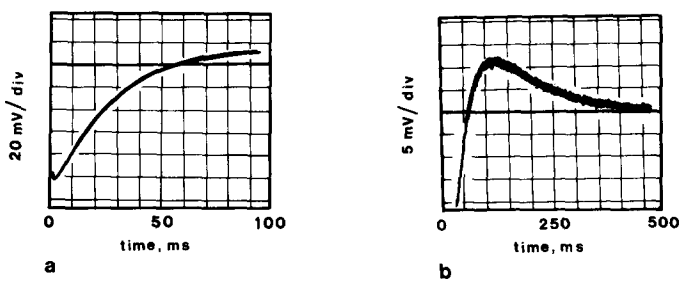


Fig. 5a and b. Oscilloscope traces at 570 nm and 5°C with the signal amplitude ΔI (mV) as a function of time (ms). The absorbance change ΔA is calculated from $\Delta A = -\log(\Delta I/I + 1)$ where I is 150 mV.

We also investigated the influence of laser intensity and measuring light intensity on the overshooting absorption in order to see if this might be due to some stray impurity or a two-proton absorption process, etc. However, all our experiments showed that the overshoot signal is not an experimental artefact, but originates from an intermediate in the photocycle of light-adapted bacteriorhodopsin. The difference spectrum in Fig. 7 was obtained by extrapolating the absorption decay to $t = 0$ assuming first-order kinetics at different wavelengths (490—610 nm). It is very similar to the absorption spectrum of BR with an absorption maximum at approx. 570 nm. Since the intermediates K-BR and L-BR have lifetimes much shorter than 1 ms at ambient temperatures and the O-form absorbs strongly at much longer wavelengths [1,5], the only species we know of that could give rise to the overshoot is pseudobacteriorhodopsin (P-BR). The excitation spectrum of this strongly emitting photoproduct of BR has a maximum at approx. 594 nm at 77 K [12]. Unpublished work (Gillbro, T., Kriebel, A. and Wild, U.P.) shows that the decay of P-BR into BR has a small activation energy of approx. 2.3 kcal/mol obtained in neutral aqueous solution. Extrapolation of the rate constant for the decay at $T \approx 85$—100 K (Gillbro, T., Kriebel, A. and Wild, U.P., unpublished) to $T = 298$ K gives a calculated lifetime of approx. 42 ms, which is to be compared to the measured lifetime of 72 ms at this temperature (see Fig. 6).

An overshoot over the baseline must mean that the absorbing species P-BR has a larger extinction coefficient at 570 nm than BR. How much larger it is will, of course, depend on the concentration of P-BR. It is possible to express the overshoot in Fig. 7 as $\Delta A = [\text{P-BR}](\epsilon_P - \epsilon_{BR})$. Since ϵ_{BR} has a maximum at 570 nm and ΔA of Fig. 7 has a maximum at the same wavelength, ϵ_P must also have a maximum close to 570 nm. If a model is assumed where a part, x, of BR forms P-BR and the rest goes into the cycle one can, for instance, calculate (e.g., from Fig. 5) that the extinction coefficient (ϵ_P) for P-BR at 570 nm has to be 3.9 times larger than for BR (ϵ_{BR}) if $x = 0.1$. In order to obtain reasonable large ϵ_P, e.g., $\epsilon_P < 1.3\ \epsilon_{BR}$, x has to be >0.5. This would mean that at 5°C a large amount of BR transforms photolytically directly into

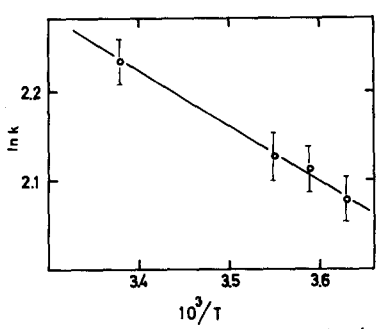


Fig. 6. Arrhenius plot of ln k (s^{-1}) for the decay of P-BR as a function of $1/T$ (K). Estimated errors are indicated (bars).

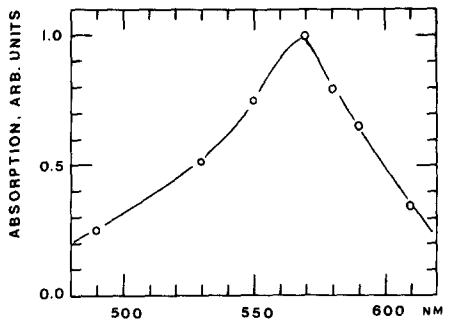


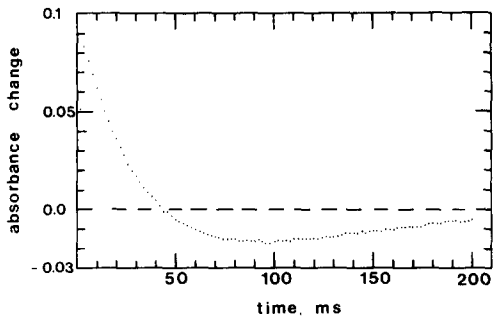
Fig. 7. The approximate absorption spectrum of P-BR at room temperature as extrapolated from kinetic data (see text). Arb., arbitrary.

P-BR and that the formation of P-BR might be temperature dependent. It is interesting to compare our results with the temperature dependence of the fast formation of a transient at 550—585 nm observed with picosecond spectroscopy [20]. It was found that the intensity of this transient increased with a factor of two to three on cooling the bacteriorhodopsin sample from room temperature to 68 K.

Another possibility, is that P-BR might also be the last intermediate in the photocycle of BR. P-BR would then be formed from O-BR and then decay into BR. The maximum of the absorption curve would correspond to the time when all O-BR has decayed into P-BR if $k_O >> k_P$. However, since $k_O/k_P = 4.66$ one can get only a rough estimate of ϵ_P at 5°C from Fig. 5. Our estimate gives $1.28\,\epsilon_{BR} > \epsilon_P > 1.14\,\epsilon_{BR}$, with $\epsilon_{BR} = 58\,000\ mol^{-1} \cdot cm^{-1}$ [18] at 570 nm it follows that $\epsilon_P = 66\,000 - 74\,000\ mol^{-1} \cdot cm^{-1}$ at 5°C. At this point it should be mentioned that at 5°C and 420 nm the transient shown in Fig. 8 was obtained. One can clearly see how M-BR decays with $t_{1/2} = 13.6$ nm. However, it does not decay to the zero line but to $\Delta A_{420} = -0.017$, i.e., also exhibits an 'overshoot'. The same effect has been noted by Vsevolodov and Kayushin [8]. There must thus exist a long-lived species which has a smaller extinction coefficient than BR at 420 nm. Since the species has a half-life of approx. 80 ms at 5°C (see Fig. 8) we believe that this species is also P-BR. The low extinction coefficient of P-BR at 420 nm as compared to BR and the larger extinction coefficient at 570 nm are consistent if the strong optical transition around 570 nm borrows its strength from the transition around 420 nm.

pH-dependence

The decay of the intermediates M-BR and O-BR together with the recovery of BR were studied at pH 3—10 at different temperatures (27—49°C). It was, however, difficult to detect any characteristic trends, which would give a simple answer to the question of at what step in the reaction cycle the depro-



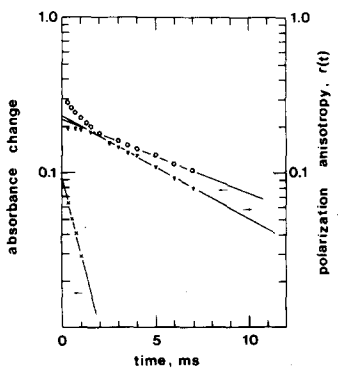


Fig. 8. The time course of the transient absorption at 420 nm and 5°C. The data points are the mean value from 16 laser flashes.

Fig. 9. The transient absorption (○) and polarization anisotrpy (▽) as studied at 570 nm and 24°C in an alkaline (pH 10) suspension of purple membrane. The transient absorption can be divided into a fast (X) and a slow (○) component.

tonation or protonation, respectively, of the membrane takes place. The decay of M-BR, for instance, was found to be almost independent of the pH in the range 7.6—3.0. In the same pH range the recovery of BR varies somewhat, but no significant change pointing in the same direction was observed. So whenever the protonation and deprotonation of the purple membranes takes place it is astonishingly independent of the pH of the surrounding medium. The O-BR transient was also almost unaffected by the pH in these acid media. We noted, however, as did Lozier and Niederberger [11], that the maximum concentration of O-BR increased as the pH decreased.

A significant change in the kinetics was, however, observed when an alkaline solution (pH 9—10) was used. In this case the decay of M-BR was composed of two first order reactions as shown in Fig. 9. The same kinetics was observed also for the recovery of BR. The formation of O-BR was very fast and the decay also showed a composite structure.

The complex kinetics observed at pH 9—10 have also been observed in media with high salt concentrations [5—7,10]. Since high Na^+ concentration alone influences the kinetics of M-BR and BR to deviate from a single first-order reaction, we cannot exclude that some of the pH effect reported here comes from Na^+ ions. In our experiments at pH 9—10 the polarization anisotropy, $r(t)$, changed drastically as compared to the neutral or acid suspensions. As shown in Fig. 9, the relaxation time at 24.2°C is only 5.5 ms as compared to the much longer time measured in neutral suspensions [11].

One could thus suspect that the biphasic kind of kinetics observed at pH 9—10 is linked to a change in the structure of the purple membrane itself. Since destruction of the chemically rather stable membranes [1] at pH 9—10 seems unlikely, a reasonable explanation for the short relaxation time might be that the dimensions of the membranes are smaller by folding or that disaggregation of aggregated purple membranes occurs at pH 10. This would increase the speed of rotational diffusion [21].

A model for the reaction cycle of bacteriorhodopsin

In this section we will propose a model which can explain the results obtained in this work and which will also have the potential to comprise some recent results on rhodopsin [22—26]. A schematic reaction cycle is shown in Fig. 10, in which the suggested elementary reaction (proton transfer and/or conformational change) of each reaction step is indicated. As a starting point we take as a chromophore the unprotonated Schiff base all-*trans* retinal (indicated by R). The reasons for this choice are: (a) the recent ^{13}C-NMR results on bovine rhodopsin [22] that indicate an unprotonated Schiff base

TABLE II

Reaction	Activation energy (kcal/mol)
L-BR → M-BR	10.7 ± 0.5
M-BR → O-BR	13.1 ± 0.5
O-BR → BR (P-BR)	3.6 ± 1.0
P-BR → BR	1.2 ± 0.4

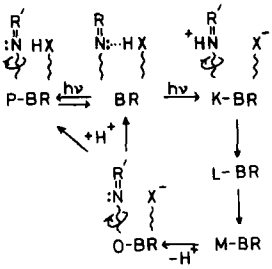


Fig. 10. A schematic model for the photoinduced reaction cycle of bacteriorhodopsin partly based on experimental result obtained in this study. R stands for the all-*trans*-retinal configuration of light adapted bacteriorhodopsin (BR) and R′ represents other configurations of all-*trans*-retinal in the photocycle.

chromophore and (b) the current belief that the all-*trans* form (in a strained configuration) constitutes the *batho*-form of rhodopsin [23—25] besides being the form of light-adapted bacteriorhodopsin. HX stands for a proton-donating group interacting with retinal.

We obviously need a model for the initial photoreaction of BR, where both K-BR [1] and P-BR [12] can be produced, and which will include the possibility of both proton transfer [26] and isomerization [23]. As illustrated in Fig. 10, K-BR might be formed by simultaneous protonation of the Schiff base and configurational change in order to prevent a reversible deprotonation. The proton being transfered is believed to interact strongly with the lone-pair orbital of the nitrogen atom through a hydrogen bond. The formation of P-BR is thought to be just a configurational change. The decay of K-BR into L-BR and the following decay of L-BR into M-BR are not supposed to involve any proton release, but only a configurational change.

As mentioned above, we have arrived at the conclusion that the proton is lost in the M-BR → O-BR reaction.

From the reaction scheme in Fig. 10 it follows that the proton might be taken up by the anion again either before of after the R-group has relaxed to its original configuration. In the first case P-BR and in the second case BR will be formed. It is likely that first reaction dominates at lower temperatures when the configurational change is supposed to be relatively slow.

The final step in the reaction sequence at low temperatures is the thermal decay of P-BR into BR. The very low activation energy (approx. 1.2 kcal/mol) of this reaction indicates that it is not a *cis-trans* isomerization around a double bond but rather a rotation around single bonds, perhaps of the kind suggested by Warshel [24].

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