PRIMARY INTERMEDIATES IN THE PHOTOCHEMICAL CYCLE OF BACTERIORHODOPSIN

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ABSTRACT Picosecond studies of the primary photochemical events in the light-adapted bacteriorhodopsin, bR_{570}, indicate that the first metastable intermediate K_{610} is formed with a rise time of 11 ps. Difference spectra obtained at 50 ps after excitation show that K_{610} is the same species as that trapped in low temperature glasses. A precursor species (S) of the K_{610} intermediate has been observed which is red shifted with respect to K_{610} and is formed within the 6-ps time width of the excitation pulse. The formation of the precursor has no observable thermal dependence between 298° and 1.8°K. The formation of K_{610} has a very low thermal barrier and at very low temperatures, the rate of formation becomes practically temperature independent which is characteristic of a tunneling process. The rate of formation of K_{610} has a moderate deuterium isotope effect of $k_H/k_D \sim 1.6$ at 298°K and 2.4 at 4°K. The mechanism for formation of K_{610} is found to involve a rate-limiting proton transfer which occurs by tunneling at low temperatures.

INTRODUCTION

Bacteriorhodopsin is produced by the *Halobacterium halobium* in response to oxygen starvation. Under these conditions, special purple membrane plaques of bacteriorhodopsin are synthesized (1–3). This simple membrane protein of 26,000 mol wt has some analogies to the visual photoreceptor rhodopsin in that both have a retinaldehyde chromophore attached as a Schiff base to a lysine residue of the protein moiety (1, 4); both produce a red-shifted metastable intermediate after the absorption of a photon; prelumirhodopsin PL_{545} is formed upon photolysis of rhodopsin (5–7); K_{610} is formed upon photolysis of light-adapted bacteriorhodopsin (8, 9). It is thus of interest to compare the initial photochemical processes in these two species.

In a study of the initial photochemical events for rhodopsin, we were able to demonstrate that prelumirhodopsin is formed by a rate-limiting proton transfer at low temperatures by a tunneling mechanism (10). Here we have extended these types of studies to the investigation of K_{610} which is formed upon excitation of light-adapted bacteriorhodopsin. We have observed that the K_{610} intermediate is formed with a rise time of about 11×10^{-12} s (11 ps) at room temperature in agreement with the results of Kaufmann et al. (11, 12). The K_{610} intermediate arises from a red-shifted transient formed within the lifetime of the photolyzing pulse (6 ps). Low temperature studies of bacteriorhodopsin and deuterium-exchanged bacteriorhodopsin indicate that the formation of K_{610} metastable intermediate is consistent with a proton transfer.

EXPERIMENTAL METHODS

Purple membranes (bR, bacteriorhodopsin) were isolated from a *Halobacterium halobium* R1-L3 strain, which was grown in a hypertonic medium following the method described by Becher and Cassim (13). The purified purple membrane fragments were characterized by sodium dodecyl sulfate gel electrophoresis which showed a single protein band of 25,000 mol wt and by its spectral absorption at 280 and 565 nm which gave an OD ratio of 2.5:1.0. The membranes were suspended in 0.01 M potassium phosphate buffer, pH 7, to achieve a final absorbance at 570 nm of 9.0.

The deuterated bacteriorhodopsin samples were prepared by suspending the fragments in D_2O at room temperature and illuminating them with a 100-W lamp. After 1 hr, the sample was centrifuged; this procedure was repeated three times. The deuterated samples were finally suspended in D_2O to give an absorbance at 570 nm of 15. Before kinetic experiments were conducted, the samples were light adapted for 20 min.

The kinetic measurements were carried out in optical cells with 2-mm path length. Low temperature studies were performed as previously described (10) with optically clear glasses from one part bacteriorhodopsin and two parts freshly distilled ethylene glycol. For deuterated samples, deuterium-exchanged ethylene glycol was used.

Measurement of spectral changes as a function of time were performed with a double-beam picosecond spectrometer and data processing system as described by Netzel and Rentzepis (14). All samples were excited at 530 nm with a single 6-ps pulse, about 2 mJ in total energy. Particular care was taken to work in a region where the response was linear with energy absorbed. We estimate about 25% of the sample was bleached per excitation pulse. Experiments with varying excitation energy assured us that the sample was not saturated under the conditions described.

RESULTS

Kinetic transients formed upon photolysis of light-adapted bacteriorhodopsin (bR_{570}) were observed over the wavelength range of 420–700 nm (Fig. 1). The time-resolved spectra of two intermediates and the population changes of bR_{570} were monitored at room temperature 20±5°C. Within the 6-ps excitation pulse width, a transient is formed which is observed to decay with a 11-ps lifetime as shown at a wavelength 660 nm (Fig. 1, 660 nm). The transient does not decay back to the ground state bR_{570} as seen in Fig. 1 (500 nm). Time-resolved spectral changes between 550 and 620 nm suggest that another metastable species is formed with a rate equal to the decay of the first transient. At 620 nm (Fig. 1) an instantaneous increase in absorbance to OD = 0.43 is observed which remains constant throughout the monitoring period of 300 ps. This observation is consistent with the assignment of an isosbestic point between the first transient and the longer-lived second intermediate. Inasmuch as the decay rate of the first transient and the formation rate of the second intermediate are equivalent, it is suggested that the first red-shifted transient (S_1) decays to form a relatively stable intermediate (K). bR_{570} is depleted upon excitation at 530 nm as observed at 500 and

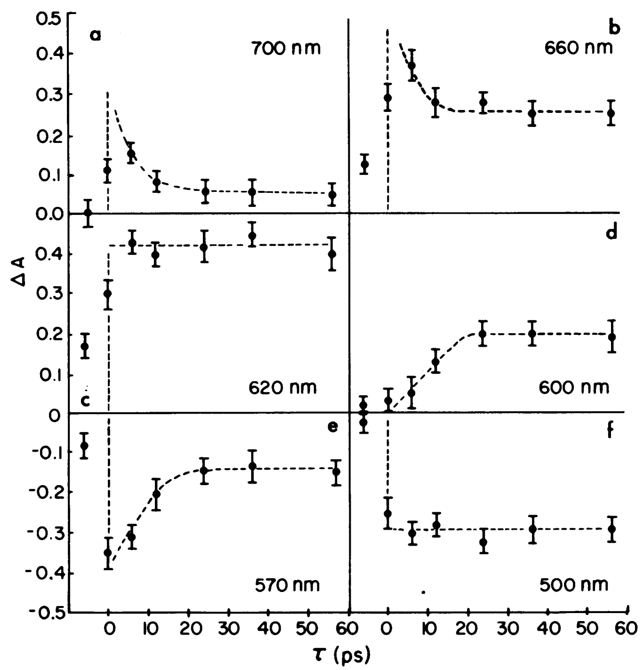

FIGURE 1 Kinetic transients observed at various wavelengths after excitation of light-adapted bR_{570} at 530 nm, 20±5°C. The excitation pulse width is 6 ps and has 2 mJ in total energy. (a) 700 nm; (b) 660 nm; (c) 620 nm; (d) 600 nm; (e) 570 nm; (f) 500 nm.

480 nm, and the change remains constant during the interrogation time of 300 ps. No optical density changes were observed at 420 nm.

The changes in absorbance as a function of wavelength at 6 and 50 ps are plotted in Fig. 2. The two difference spectra illustrate that the initial transient is red-shifted compared with the starting bR_{570}, and this transient then decays to a less red-shifted second intermediate. By normalizing the spectrum for the starting bR_{570} concentrations (and assuming that the observed absorption decrease at 480 and 500 nm are solely due to bleaching of bR_{570}), the absorption spectra of each of these new red-shifted intermediates may be calculated as depicted in Fig. 3. The initial transient has a λ_{max} of 625 nm (termed S), and the second intermediate has a λ_{max} of 615 nm (termed K). The spectra do not reflect the S decay during the first 11 ps.

The temperature dependence of the rate of formation of the new intermediates after excitation of bR_{570} was monitored at 570 nm. The rate of formation of K increases from 11 ps at 295°K to 52 ps at 1.8°K. When the data are plotted in an Arrhenius manner (ln k vs. $1/T$; Fig. 4), they do not behave in a classic manner but show a nonlinearity and indicate that the formation of K proceeds at a finite rate as the temperature approaches 0°K.

To investigate the role of proton translocation in these early photoinduced intermediates, deuterium-exchanged bR_{570} was prepared and the kinetics of the transients

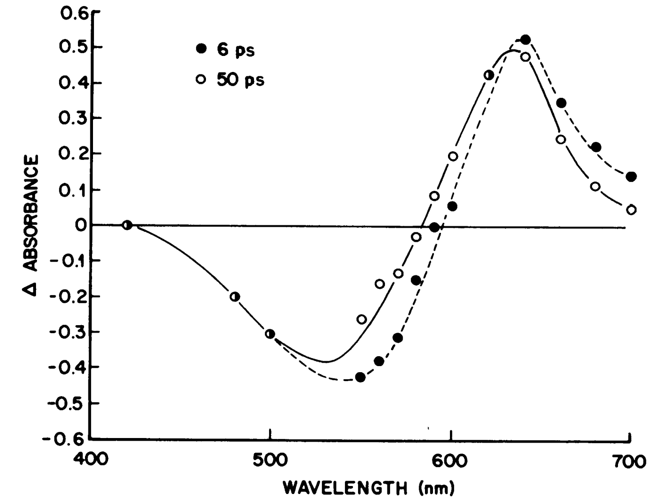

FIGURE 2 Difference spectra of bR_{570} and the intermediates observed at 6 and 50 ps after excitation with a pulse at 530 nm. The amplitudes are normalized to a starting absorbance of $OD_{570} = 3$ for a 2-mm path length. (●) Amplitudes at 6 ps; (○) amplitudes at 50 ps.

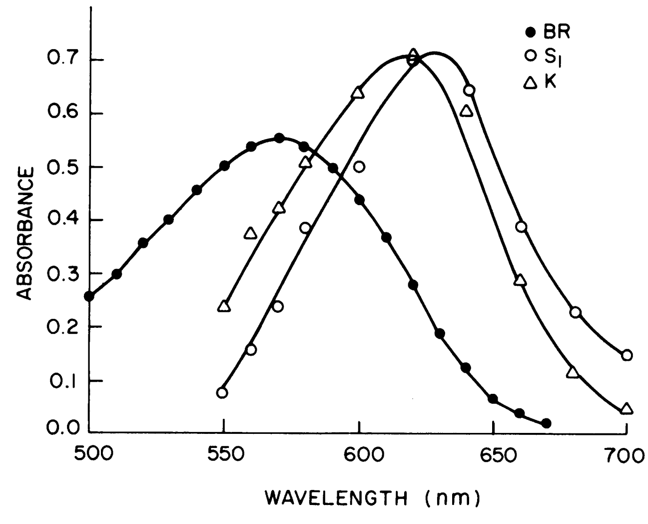

FIGURE 3 Absorption spectra for light-adapted bR_{570} and transient intermediates S_1 and K. The spectra are calculated from a normalized spectra for bR_{570} and the values given by the difference spectra in Fig. 2. (●) bR_{570}; (○) transient present at 6 ps (S_1); (Δ) intermediate present at 50 ps (K). Note that such calculated spectra are qualitatively correct but are not necessarily quantitatively correct.

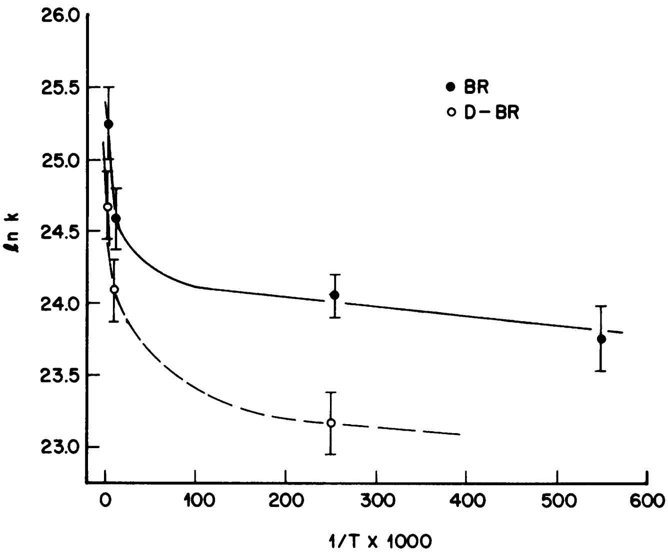

FIGURE 4 Temperature dependence of rate of formation of K. (•) Aqueous-suspended bacteriorhodopsin; (o) deuterium-exchanged bacteriorhodopsin.

were observed as discussed above. Deuteration has no observable effect upon the rate of formation or amplitude of the initial transient formed either at room temperature or low temperatures. The decay of this transient at room temperature, and hence the formation of K, is decreased, however, from 11 ± 2 to 18 ± 3 ps. At low temperatures, the rate is also decreased, e.g., at 4°K, K is formed in 36 ps, deuterium-exchanged K is formed within 88 ps. Thus a moderate deuterium isotope effect of 1.6–2.4 is observed for the formation of the K intermediate. The temperature dependence of the formation of deuterium-exchanged K is plotted in Fig. 4.

DISCUSSION

The previous studies of Kaufmann et al. (11, 12) have indicated that a metastable intermediate, K, is formed within 15 ps upon excitation of bacteriorhodopsin, bR_{570}. Their studies did not encompass a wide range of wavelengths, and their sensitivity was decreased because of low concentrations of sample. We have thus extended the studies of bR_{570} to include a full survey of transients over the visible wavelength range, a description of the temperature dependence of the rate of formation of intermediates, and the effect of deuteration on the formation of these early intermediates.

In agreement with the data of Kaufmann et al. (11, 12), we detect an initial transient, termed S, which is formed within the excitation pulse width, and a second intermediate, K, which is formed within 11 ± 2 ps. The latter is relatively long lived with a lifetime >300 ps, the extent of our measurements.

The difference spectra of the transients and bR_{570} depleted upon excitation presented at 6 and 50 ps (Fig. 2) indicate that the initial transient, S, is maximally red shifted with respect to the starting bR_{570} and decays within 11 ps. The second relatively long-lived intermediate, K, is formed within 11 ps and is less red shifted, showing a maximum at 615 nm (Fig. 1–3). Because the decay of S and formation of K are equivalent and an isosbestic point at ~620 nm between these two intermediates is observed, we have assumed S decays to form K. The relatively long-lived K intermediate detected here at room temperature is equivalent to the K_{610} intermediate previously observed in studies of low temperature glasses (8, 9). Kinetic studies of this intermediate in the microsecond range indicate a lower λ_{max} at 590 nm (K_{590} nm, references 8, 9, 15).

The rates of formation of these intermediates were studied as a function of temperature in optically clear glasses to further elucidate the mechanism by which these species arise. The rise time of the initial transient S is not resolved, even at the low temperature of 1.8°K. In the case of the formation of K, we find the rise time increases from 11 ps at 295°K to 20 ps at 77°K which is equivalent to an activation barrier of 140 cal/mol. This is indeed lower than thermal energy of 600 cal/mol available at room temperature (298°K). Below 77°K the formation of K appears to be achieved by an alternative mechanism as shown in Fig. 4. The rate of formation is practically temperature independent and suggests that a tunneling process is functional at low temperatures and may be a process even at higher temperatures. The most likely candidate for this process is a proton, as has been suggested for similar observation in the study of rhodopsin (10).

To experimentally confirm that the production of K involves proton translocation, the purple membrane particles were deuterium exchanged and the kinetics of formation of K were reexamined. Deuterium isotope effects have been observed for the formation of each of the other intermediates in the bacteriorhodopsin cycle (16), and we have now observed an effect for the formation of the first metastable intermediate as well. At 298°K the deuterium isotope effect for formation of K, $k_H/k_D = 1.6$, is consistent with the difference in zero-point energy between —X—H and —X—D vibrations which would affect a thermally activated process. At 4°K, where the proton translocation process proceeds by tunneling, the ratio k_H/k_D becomes ~2.4. The effect is less than that observed for the formation of prelumirhodopsin at 4°K ($k_H/k_D = 7$) and suggests that rhodopsin has a greater energy of activation (height and/or width of the barrier) for proton translocation than bacteriorhodopsin.

In a previous paper on proton tunneling in rhodopsin (10), we employed an expression for the rate of tunneling, as given by Löwdin (17), where the energy of activation can be calculated based on the experimental rate constant, an assumed frequency factor for the N—H vibration, and a distance for proton transfer. With a rate constant of 2.8×10^{10} s, a frequency of 1,500 cm^{-1} for N—H, and a distance of 0.9 Å, a value of 1.4 kcal/mol was calculated for the energy of activation. If instead of using the N—H frequency, we use the frequency of a proton in a hydrogen bond (18), this would result in a lower barrier for activation of 740 cal/mol for rhodopsin. These values represent limits for the barrier height based on present tunneling theories (17–19), which do not necessarily describe the barrier shape and other conditions of the biological system. We would expect that the energy of activation for a proton transfer in bacteriorhodopsin to be somewhat <740 cal/mol based on its smaller deuterium isotope effect.

Ebrey, Rosenfeld, and their colleagues have recently proposed that the primary photochemical event in bacteriorhodopsin, as well as rhodopsin, is cis-*trans* isomerization of the respective retinal chromophores (20,21). They propose that bR_{570} and the K intermediate share a common excited state in which isomerization occurs by a barrierless transition. Experimental evidence supporting the common excited-state hypothesis is based on their studies of quantum yield for the process. They have determined a quantum yield $\phi_1 = 0.30$ for the formation of K and from the ratios of the quantum yields ϕ_1/ϕ_2 measured under conditions of photostationary equilibrium for the forward and backward processes $bR_{570} \underset{\phi_2}{\overset{\phi_1}{\rightleftarrows}} K$, they establish the quantum yield of the back reaction $\phi_2 = 0.70$ (22, 23). Because $\phi_1 + \phi_2 = 1.0$, the quantum yield studies support the common excited-state hypothesis.

Based on these quantum yields and our observed lifetime of 11 ps for the formation of K from the transient state S, we would expect to observe repopulation of the ground state bR_{570} at 490 nm corresponding to the 70% recovery of the initially depleted bR_{570}. Such a repopulation would have a similar lifetime of 11 ps. We observe, however, that within the 6-ps excitation pulse there is an optical density decrease at 490 nm and there is no further change in this optical density throughout the following interrogation time of 300 ps. If S_1 is assigned to the first excited singlet, we can state that no observable internal conversion occurs from S_1 back to bR_{570} to account for the appropriate quantum yield. We therefore conclude that photoreversion of K to bR_{570} proceeds through a state other than S_1 of bR_{570}. Our data present a challenge to the concept of a common excited-state hypothesis and suggest that modification of the photochemical mechanism is needed to accommodate both sets of data.

This research was supported in part by a grant from the National Eye Institute (EY-01542) to M.L.A.

Received for publication 19 February 1978 and in revised form 5 May 1978.

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