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ABSORPTION SPECTRA OF INTERMEDIATES OF BACTERIORHODOPSIN MEASURED BY LASER PHOTOLYSIS AT ROOM TEMPERATURES

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Picosecond laser spectroscopic analysis was applied to determine how many intermediates existed in the primary photochemical process of *trans*-bacteriorhodopsin (light-adapted bacteriorhodopsin) at room temperature (18°C) and to calculate their absorption spectra. Irradiation of bacteriorhodopsin with a laser pulse (wavelength, 532 nm; pulse width, 25 ps) yielded the K intermediate (K) which was produced through a precursor, having an absorption maximum (λ_{max}) longer than that of K. K was stable during a picosecond time range (50–900 ps). The λ_{max} was located at 610 nm and the extinction coefficient (ϵ_{max}) was 0.92-times that of bacteriorhodopsin. The same K intermediate was produced from bacteriorhodopsin even when it was excited with a high-energy pulse by which a saturation effect was induced. A transient difference spectrum measured at 150 ns after the excitation of bacteriorhodopsin was different in shape from that of the K intermediate, suggesting that an intermediate was formed by thermal decay of K. This intermediate, tentatively called the KL intermediate (KL), had a λ_{max} at 596 nm and an ϵ_{max} 0.80-times that of bacteriorhodopsin. KL decayed to the L intermediate (L) with a time constant of 2.2 μs. L has a λ_{max} at 543 nm and an ϵ_{max} 0.66-times that of bacteriorhodopsin.

Introduction

Bacteriorhodopsin is a photoreceptive pigment present in the purple membranes of *Halobacterium halobium* [1]. It has two physiological forms; light-adapted and dark-adapted bacteriorhodopsins. The light-adapted bacteriorhodopsin has an all-*trans*-retinal as its chromophore, while dark-adapted bacteriorhodopsin is an equilibrium mixture of two isomeric forms which have all-*trans*- and 13-*cis*-retinals (1 : 1) as their chromophores [2–4].

On irradiation of light-adapted bacteriorhodopsin at liquid nitrogen temperature, it converts to a bathochronic photoproduct called bathobacteriorhodopsin [5] which may correspond to K [6], R_2 [7] and ^{630}T [8], named by respective research groups on the basis of low-temperature spectrophotometry or flash photolysis at relatively low temperatures. It has been reported that K was also detected by use of picosecond and nanosecond laser photolyses at room temperatures [6,9–11]. However, there are some discrepancies in the absorption spectrum of K between picosecond [11] and nanosecond [6,9] laser photolytic experiments. A picosecond experiment showed that K has a λ_{max} at 615 nm which is 25 nm longer than that estimated by a nanosecond experiment [6]. In order to clarify the discrepancy between these results, we have undertaken to measure the transient absorbance changes of bacteriorhodopsin after excitation by a picosecond laser pulse at room temperature, and found that at least four intermediates existed in a time range from 0 ps to 5 μs.

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Materials and Methods

Preparation of bacteriorhodopsin in purple membrane. Purple membranes from *H. halobium* R1-M1 strain were isolated by a sucrose linear gradient method described by Iwasa et al. [5] and then suspended in distilled water. The suspensions were sonicated for 45 s and then adjusted to pH 8.0 by adding $NaHCO_3$ or by adding 20 mM phosphate buffer.

An optical system for picosecond absorption measurements. The optical system is basically similar to that described previously [12,13] except that an Nd^{3+}-YAG laser (Matsui Co. Ltd.) was used for exciting the sample. A train composed of picosecond fundamental pulses (1064 nm, 25 ps width) was generated by a mode-locked Nd^{3+}-YAG laser. A single and bright pulse was isolated from the pulse train by a single-pulse selector and then amplified to about 30 mJ by two sets of amplifiers. The pulse was focused on a phase-matched KDP crystal for generating a frequency-doubled green-light pulse (532 nm, 0.5 mJ). The 532 nm pulse thus obtained was separated from the fundamental pulse by a beam splitter and focused on a sample cell after passing through a movable (delay) prism. The fundamental pulse (about 25 mJ) was focused into a glass block to generate a picosecond continuum, which was used as a monitoring pulse. The picosecond continuum was focused on a diffuser (ground glass) for diminishing an inhomogeneity of distribution of light intensity and then split into two beams by a half mirror; one (s) was used for monitoring an absorption change of the sample and the other (r) for checking the intensity and correcting the spatial distribution of the monitoring pulse.

A movable prism was used for changing an arrival time of the 532 nm excitation pulse to the sample in order to measure the laser-induced absorption changes at a required time after the excitation. The zero time, which means that both excitation and monitoring pulses reach the sample at the same time, was checked by use of a CS_2 Kerr optical shutter [14].

During the experiment, the energy of the excitation pulse was monitored by a biplanar photodiode (Hamamatsu Co., Ltd.). Then the energy was calibrated by that measured on the surface of the sample cell by a joule-meter (GEN-TEC Inc.) after the experiment.

Monitoring pulses were focused on a slit of a polychrometer (Jarrel Ash Co., Ltd.) and detected by a Vidicon target (ISIT, PAR Co., Ltd.) combined with an OMA 2 system (PAR Co., Ltd.).

In order to calculate a difference spectrum between bacteriorhodopsin and its intermediates, intensities of both the monitoring pulses (I_s and I_r) were measured with (ex) and without (no) excitation of the sample and then the difference spectrum was calculated according to the following equation:

$$A = -\log_{10}\left[\frac{I_s^{ex}}{I_r^{ex}} / \frac{I_s^{no}}{I_r^{no}}\right]$$

An optical system for nanosecond absorption measurements. In this system, the 532 nm picosecond pulse from the mode-locked Nd^{3+}-YAG laser was used for excitation of the sample, and a photographic flashlamp (Matsushita Co., Ltd.) was used as a monitoring light. The Vidicon target combined with the OMA 2 system was used as a detector which was gated by a high-voltage pulse generator (PAR Co., Ltd.) in order to measure the absorbance change of the sample at an adequate time after the excitation.

For measuring nanosecond kinetics after excitation of bacteriorhodopsin, a photomultiplier tube (Hamamatsu Co., Ltd.) combined with a storage oscilloscope (7834, Tektronics Co., Ltd.) was used.

The sample was light adapted with orange light ($\lambda > 500$ nm) for more than 20 min just before the experiment. Absorbance of the sample (10 μl) was 0.7–1.0 in an optical cell (2 mm optical path and 2 mm width).

In order to check the reliability of the absorption spectra measured by use of both picosecond and nanosecond optical systems, the absorption spectra of bacteriorhodopsin measured by these systems were compared with that measured by use of a conventional spectrophotometer (Shimazu, MPS 5000). They were in good agreement with one another.

Results

A series of difference spectra between bacteriorhodopsin and its intermediates was successively

measured in the range from 0 ps (just after the excitation) to 500 ps after the excitation of bacteriorhodopsin. As shown in Fig. 1, a bathochromic product was observed even at 0 ps (curve 1). The difference spectrum measured at 0 ps (curve 1) was located at about 10 nm longer wavelengths than the other spectra (curves 2–4) which were almost identical with one another within the experimental resolution. Thus, we infer that there may exist two bathochromic intermediates in this time range. Formation and decay processes of the earlier intermediate could not be measured because of our instrumental limitation. We shall call the earlier intermediate the J intermediate (J) and the later intermediate K intermediate (K).

A difference spectrum at 900 ps after the excitation was also measured (data not shown). Its shape was in good agreement with those of curves 2–4 in Fig. 1, indicating that K is stable in at least the picosecond range.

The multiphoton effect due to the high energy of the excitation pulse was examined by measuring the difference spectra between K and bacteriorhodopsin at 100 ps after the excitation as a function of intensity of the excitation pulse. In Fig. 2 absorbances at 630 nm from the difference spectra were plotted against intensities of excitation pulses. The information of K linearly increased until the excitation energy reached 14 μJ and above this energy the increase in absorbance was saturated. The difference spectra obtained by excitation with various energy pulse are shown in Fig. 3, where all the difference spectra are almost identical in shape with one another even when bacteriorhodopsin was excited with a high-energy pulse. The ratios of $\Delta A_{620\ nm}$ to $\Delta A_{550\ nm}$ in the difference spectra measured by exciting with 3, 7.2 and 22.4 μJ pulses were 1.31 ± 0.2, 1.37 ± 0.2 and 1.30 ± 0.2, respectively.

Fig. 4 shows the difference spectra measured at 100 ps, 150 ns, 1 μs and 5 μs after the excitation of bacteriorhodopsin. Since curve 1 is the difference spectrum between K and bacteriorhodopsin, the change of curve 1 to curve 2 reflects the decay of K. However, the difference spectrum between curves 1 and 2 was different in shape from those between curves 2 and 3 and between curves 3 and 4. The latter were almost identical in shape with each other. Therefore, the spectral change from curve 2 to curve 4 may reflect a decay process of an intermediate which has converted from K. An absorption spectrum (curve 4 in Fig. 5a) of an

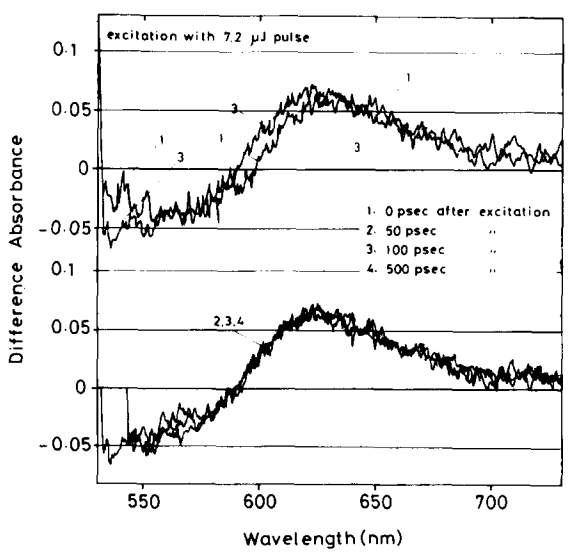

Fig. 1. Difference spectra between bacteriorhodopsin and its intermediate measured at 0, 50, 100 and 500 ps (curves 1–4) after excitation of bacteriorhodopsin with a laser pulse (wavelength, 532 nm; duration, 25 ps). Each spectrum represents the average of 20 experiments.

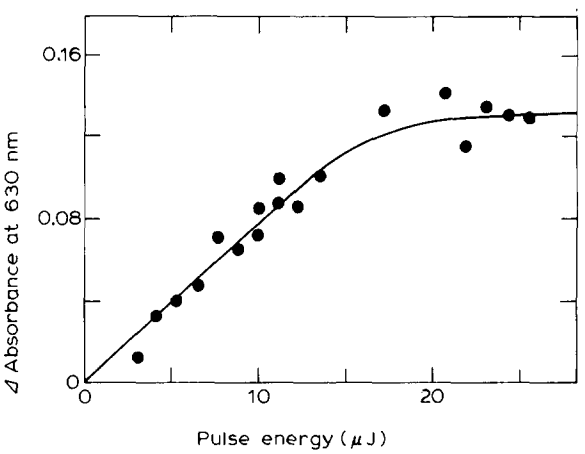

Fig. 2. Relationship between change in difference absorbance at 630 nm and laser intensity. Difference spectra between K and bacteriorhodopsin were measured at 100 ps after the excitation as a function of laser pulse energy and then change in absorbance at 630 nm was plotted as a function of laser intensity. Each point represents the average of four to six experiments.

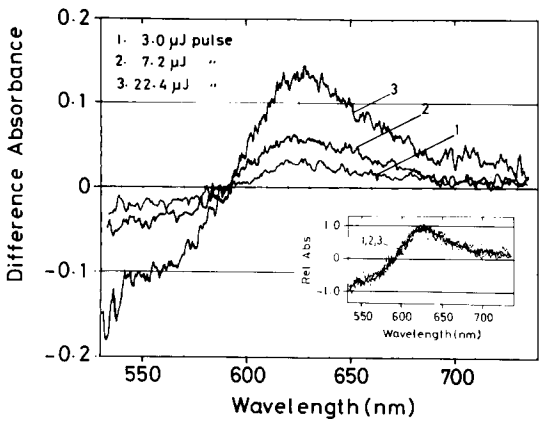

Fig. 3. Difference spectra between K and bacteriorhodopsin measured at 100 ps after excitation of bacteriorhodopsin with 532 nm light pulses of 3.0, 7.2 and 22.4 μJ (curves 1–3). (Inset) These spectra were normalized at difference maxima (625 nm)

intermediate which was converted from the above-described intermediate is similar to that of the L intermediate (L) which had been measured by means of low-temperature spectrophotometry [5] and nanosecond laser photolysis [6,9]. In fact, the formation time of this intermediate was estimated to be 2.2 μs by means of nanosecond kinetic experiments (data not shown). This value is close to that reported by Lozier et al. [6]. Although the decay process from 100 ps to 150 ns could not be measured because of our instrumental limitation, there would exist at least two intermediates in this

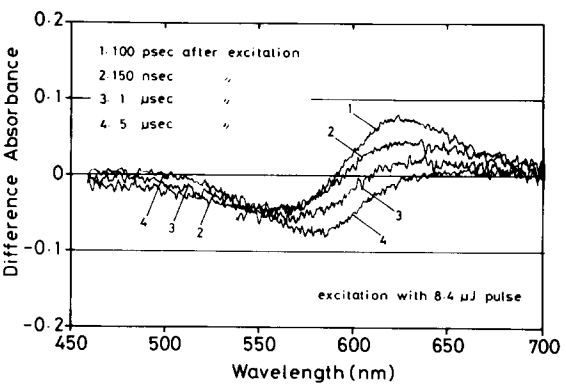

Fig. 4. Difference spectra of bacteriorhodopsin and the intermediates measured at 100 ps, 150 ns, 1 μs and 5 μs (curves 1–4) after the excitation of bacteriorhodopsin with a 532 nm light pulse. Each spectrum represents the average of 20 experiments.

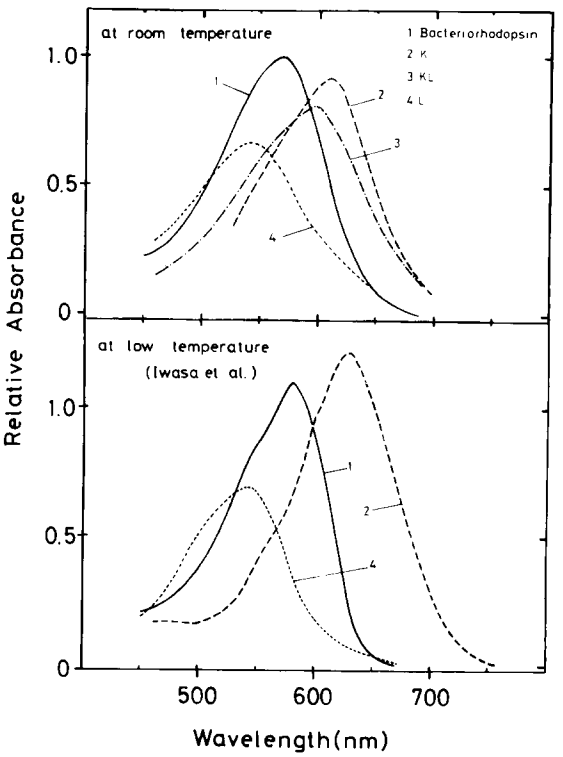

Fig. 5. Absorption spectra of bacteriorhodopsin and its intermediates at room (18°C, top) and low (bottom) temperatures. (1) Bacteriorhodopsin, (2) K, (3) KL, (4) L. Absorption spectra of bacteriorhodopsin and K in b were measured at −190°C and that of L was measured at −90°C by means of conventional low-temperature spectrophotometry [5].

process. We shall tentatively denote the intermediate detected at 150 ns after the excitation as the KL intermediate (KL), because it lies between K and L intermediates, and assume that K decays to KL.

For calculating the absorption spectrum of an intermediate, the percentage of conversion of original bacteriorhodopsin to the intermediate by excitation with the laser pulse must be estimated. A difference spectrum between the M intermediate (M) and bacteriorhodopsin was measured at 1 ms after the excitation, because M has no absorbance at wavelengths longer than 560 nm [5]. Thus, the percentage was calculated from the ratio of the absorbance at 570 nm of the measured difference spectrum to that of the original bacteriorhodopsin. When bacteriorhodopsin was excited by an 8.4 μJ

pulse, 16.3% of bacteriorhodopsin was converted. This percentage is applicable to the earlier intermediates, because we confirmed that all the molecules of the intermediates changed to M at room temperatures [15,16]. Formation of the O intermediate (O) was not observed under our experimental condition.

The absorption spectrum of K was calculated from curve 1 in fig. 4. Since 16.3% of the original bacteriorhodopsin was converted to K by excitation with the 8.4 μJ pulse, the absorption spectrum of K was obtained by adding the absorption spectrum of the original bacteriorhodopsin to curve 1 in Fig. 4 which had been corrected to 100% conversion (multiplied by 1/0.163).

The absorption spectra of KL and L intermediates were calculated as follows: An absorption spectrum calculated from curve 2 was composed of absorption spectra of 93.4% KL and 6.6% L, because the decay time of KL was 2.2 μs as described above and curve 2 was measured 150 ns after the excitation of bacteriorhodopsin. On the other hand, an absorption spectrum calculated from curve 4 was composed of absorption spectra of 1.1% KL, 94.8% L and 4.1% M, because the decay time of L to M was estimated to be 79 μs (data not shown). The absorption spectrum of M was calculated from the difference spectra measured at 1 ms after the excitation of bacteriorhodopsin. Thus, the absorption spectra of KL and L were calculated by solving simultaneous equations obtained from three sets of absorbance values of the absorption spectra calculated from curves 2 and 4 and that of M.

The absorption spectrum of J could not be calculated because of its rapid decay to K.

Fig. 5a shows the absorption spectra of bacteriorhodopsin and its intermediates calculated from the difference spectra in Fig. 4. For comparison with those spectra, the absorption spectra of bacteriorhodopsin and its intermediates at low temperatures by means of conventional spectrophotometry [5] are also shown in Fig. 5b. The λ_{max} of K at room temperature lies at 610 nm which is 16 nm shorter than that at $-190°C$. The ϵ_{max} of K at room temperature is 0.92-times that of bacteriorhodopsin. Since the ϵ_{max} of bacteriorhodopsin increases 1.1-fold when it is cooled from 20 to $-190°C$, and the ϵ_{max} of K at $-190°C$ is 1.1-times that of bacteriorhodopsin at $-190°C$ [5], the ϵ_{max} of K at $-190°C$ is 1.3-times that at room temperature.

KL, which was only detected at room temperature, has a λ_{max} at 596 nm and an ϵ_{max} 0.80-times that of bacteriorhodopsin.

L has a λ_{max} at 543 nm and an ϵ_{max} 0.66-times that of bacteriorhodopsin at room temperature, while it has a λ_{max} at 543 nm and an ϵ_{max} 0.63-times that of bacteriorhodopsin at $-90°C$.

Discussion

The photochemical reaction of bacteriorhodopsin was investigated by means of picosecond laser photolysis at room temperature, and at least three intermediates, termed J, K and KL, were detected before formation of L.

Ippen et al. [17] reported a rapid increase in absorbance at 615 nm with a time constant of 1.0 ± 0.5 ps after excitation of bacteriorhodopsin by means of a subpicosecond laser pulse. According to Applebury et al. [11], this product decayed with a time constant of 11 ps and was a precursor of K. We have also observed the formation of the precursor of K (curve 1, Fig. 1), although our instrumental resolution was about 30 ps. The reason why the difference in shape between curve 1 and curve 3 in Fig. 1 should be due to the presence of an intermediate different from K and not the time dispersion [18] of our monitoring pulse is as follows: Although the time dispersion of a monitoring pulse may cause some change in the shape of the difference spectrum, the intersection point between the difference spectrum and the baseline should not be affected by it. Therefore, the difference in wavelength between the intersection point of curve 1 with the baseline and that of curve 3 with the baseline in Fig. 1 should be attributed to the presence of different intermediates.

The precursor of K is likely a ground state as discussed by Dinur et al. [19]. Therefore, we call this intermediate the J intermediate as termed by Dinur et al. [19].

The absorption spectrum of K is somewhat different in shape from that reported previously [11]. K which we have calculated has a λ_{max} at 610 nm and an ϵ_{max} 0.92-times that of bacteriorho-

dopsin, while the one which Applebury et al. [11] reported has a λ_{max} at 615 nm and an ϵ_{max} about 1.28-times that of bacteriorhodopsin. These discrepancies may be due to different estimation of the percent conversion of bacteriorhodopsin after the excitation. They estimated the percent conversion in such a way that the decrease in absorbance at about 490 nm of the difference spectrum between K and bacteriorhodopsin was compensated by adding the absorption spectrum of bacteriorhodopsin, i.e., they assumed that K has no absorbance at about 490 nm. On the other hand, we estimated the percent conversion from the amount of M produced at 1 ms after the excitation of bacteriorhodopsin. Therefore, the percent conversion they estimated was less than that we estimated.

It might be possible to consider that the amount of M produced was underestimated because of tumbling of purple membrane fragments in the sample within 1 ms after excitation. It has been reported, however, that the time constant for tumbling of the purple membrane fragment is of the order of 100 ms [20,21], although some spatial disordering occurs in the time range more than 500 μs after the excitation [21]. Moreover, we have observed a clear isosbestic point at 455 nm in the thermal conversion process of L to M (from 10 μs to 1 ms). Thus the possibility that the tumbling of the membrane fragment would occur before the formation of M is very unlikely.

As shown in Fig. 5, the oscillator strength of K at room temperature is much smaller than that at $-190°C$, while bacteriorhodopsin has almost the same oscillator strength at both temperatures. This fact may suggest that the retinylidene chromophore of K at room temperature is more distorted than that at $-190°C$, because the oscillator strength of the long-wavelength band of aromatic and conjugated systems is so sensitive to changes in the coplanarity of the system that a small deviation from planarity may cause a decrease in the oscillator strength of the system [22]. A difference in configuration of the retinylidene chromophore of K between these temperatures may cause a difference in the chromophore-opsin interaction of K between these temperatures. Therefore, KL was produced only at room temperature.

The absorption spectrum of KL is quite similar to those of the 'K intermediate' reported by Lozier et al. [6] and Goldschmit et al. [9]. The decay time constant of KL to L (2.2 μs) is also very close to those reported by those authors. Therefore, there exist at least two intermediates before formation of the K intermediate which they described.

KL, as compared with bacteriorhodopsin and K, has a broad half-bandwidth of absorption spectrum. This fact suggests that KL would be a mixture of at least two components or has a shallower potential surface in the ground state than K. If the latter were correct, some conformational change of bacterioopsin would occur during this period.

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