Absolute absorption spectra of batho- and photorhodopsins at room temperature

Picosecond laser photolysis of rhodopsin in polyacrylamide

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ABSTRACT Picosecond laser photolysis of rhodopsin in 15% polyacrylamide gel was performed for estimating absolute absorption spectra of the primary intermediates of cattle rhodopsin (bathorhodopsin and photorhodopsin). Using a rhodopsin digitonin extract embedded in 15% polyacrylamide gel, a precise percentage of bleaching of rhodopsin after excitation of a picosecond laser pulse was measured. Using this value, the absolute absorption spectrum of bathorhodopsin was calculated from the spectral change before and 1 ns after the picosecond laser excitation (corresponding to the difference spectrum between rhodopsin and bathorhodopsin). The absorption spectrum of bathorhodopsin thus obtained displayed a λ_{max} at 535 nm, which was shorter than that at low temperature (543 nm) and a half bandwidth broader than that measured at low temperature. The oscillator strength of bathorhodopsin at room temperature was smaller than that at low temperature. The absolute absorption spectrum of photorhodopsin was also estimated from the difference spectrum measured at 15 ps after the excitation of rhodopsin (Shichida, Y., S. Matuoka, and T. Yoshizawa. 1984. *Photobiochem. Photobiophys.* 7:221–228), assuming a sequential conversion of photorhodopsin to bathorhodopsin. Its λ_{max} was located at ~570 nm, and the oscillator strength was smaller than those of rhodopsin and bathorhodopsin.

INTRODUCTION

Elucidation of the molecular mechanism of the extraordinarily high photosensitivity of rhodopsin is one of the most challenging fields of visual photochemistry. Application of low temperature spectrophotometry and picosecond laser photolysis to the primary steps of photochemical reaction of rhodopsin provoked a fruitful discovery of several intermediates (1–3). The picosecond laser photolysis is superior in that it can be used for investigation of the photochemical reaction at physiological temperature (4). However, it is inevitably weak for measuring an absolute absorption spectrum which is essential for elucidating the structure of chromophore and the chromophore-protein interaction in the intermediate. In a picosecond laser system, both excitation and monitoring pulses are usually so small in diameter that all the rhodopsin molecules in the sample cell can neither be excited nor monitored. This causes difficulty in estimating an accurate percentage of bleaching of rhodopsin sample in the excited area of the sample cell, because rhodopsin molecules thus excited diffuse in the sample cell after the excitation. Because it is essential to measure the accurate percentage of bleaching for calculation of absolute absorption spectra of intermediates of rhodopsin, we have attempted to make rhodopsin molecules immobilized in polyacrylamide gel and to estimate the accurate percentage of bleaching of rhodopsin, followed by presentation of absolute absorption spectra of cattle batho- and photorhodopsins.

MATERIALS AND METHODS

Preparation of rhodopsin sample embedded in polyacrylamide gel

Rod outer segments (ROS) were separated from cattle retinas by a sucrose flotation method described previously (5). The rhodopsin in ROS was extracted with 2% digitonin in 10 mM Hepes buffer (pH 7.0). The extract was mixed with an equal volume of 30% acrylamide solution containing 0.8% bis acrylamide. After removing the gas dissolved in the mixture by use of an aspirator, the polymerization of the acrylamide was initiated by addition of 10% ammonium peroxodisulfate (in H_2O) and *N,N,N',N'*-tetramethylethylenediamine of 1/100 and 1/1,000 vol of the previous solution, respectively. Immediately, the mixture was pipetted into several sample cells. This sample cell was composed of two long ($5 \times 60 \times 2$ mm^3) and one short ($5 \times 14 \times 2$ mm^3) silicon rubbers with an interstitial space ($4 \times 60 \times 2$ mm^3) which were sandwiched between two slide glasses (Fig. 1 *a*). Using this sample cell, we prepared a long and slender 15% polyacrylamide gel ($4 \times 60 \times 2$ mm^3). After the completion of polymerization, a short silicon rubber was removed and the gel in the sample cell was put into 50 mM hydroxylamine dissolved in 10 mM Hepes buffer (final pH 7.0) and incubated for >36 h, during which the hydroxylamine was penetrated into the gel. We call this preparation "rhodopsin gel." All the experiments were done under dim red light unless otherwise described.

Examination of immobility of rhodopsin molecule in the gel

An absorption spectrum of rhodopsin gel was measured by a spectrophotometer (model 330; Hitachi Ltd., Tokyo) using an aperture of 2-mm diameter in front of the sample cell (curve 1 in Fig. 2). The sample was then completely bleached with an orange light (>560 nm) passed through the aperture. The light source of the irradiation was a tungsten-halogen lamp (1 kw; Sanko, Tokyo) which had passed through

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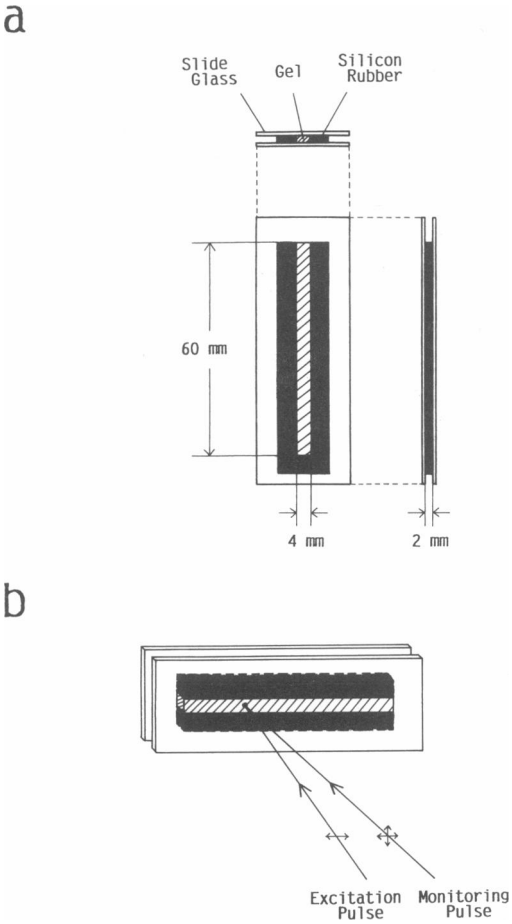

FIGURE 1 (*a*) A sample cell for the rhodopsin gel. It is composed from two slide glasses and three silicon rubbers (two long and one short). The rhodopsin sample to be polymerized was pipetted into the cell. After completion of polymerization, the short silicon rubber was removed. (*b*) Schematic drawing of the sample cell for picosecond absorption measurement. An aperture of 1.2 mm diameter was placed behind the sample cell, where a monitoring and excitation pulses were crossed each other. The monitoring pulse, which was generated from a glass block by self-phase modulation effect (SPM) of a fundamental pulse of Nd:YAG laser, was focused on a ground glass, for changing from polarized pulse into unpolarized one. The monitoring pulse was focused on the aperture behind the sample cell, whereas the excitation pulse, which was linearly polarized, was not focused. The angle between monitoring and excitation pulses was smaller than 5°. The sample cell was horizontally moved after every excitation for excitation of unexcited area of the gel.

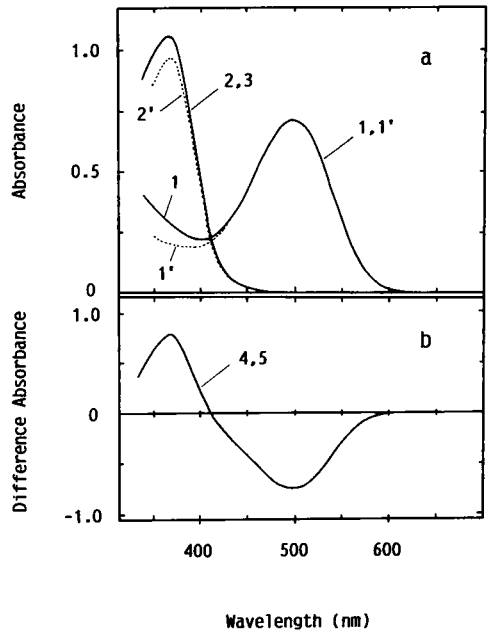

FIGURE 2 (*a*) Absorption spectra of rhodopsin gel (curve 1) and its completely bleached product measured immediately (curve 2) and 1 h (curve 3) after irradiation of orange light (>560 nm) from the steady light source. An aperture of 2 mm diameter was placed in front of the sample cell. No difference between curves 2 and 3 indicates no translational diffusion of rhodopsin molecule in the gel (see text). Curves 1′ and 2′ (*dotted line*) are spectra of rhodopsin-digitonin (2%) complex and its completely bleached product in the presence of 50 mM hydroxylamine (pH 7.0). Absorbance at λ_{max} of curve 1′ was equalized to that of curve 1. (*b*) Difference spectra between rhodopsin and its bleached product in 15% polyacrylamide gel (curve 4) and in 2% digitonin solution (curve 5). Curves 4 and 5 are differences between curves 1 and 2, and between curves 1′ and 2′.

a glass cut-off filter (VO-58; Toshiba, Tokyo). To examine translational diffusion of rhodopsin molecule in the gel, we repeatedly measured the absorption spectrum of the gel (curves 2 and 3 in Fig. 2). If the translational diffusion of rhodopsin molecule took place, the bleached molecules in the irradiated area would be mixed with unbleached molecules (rhodopsin) in the unirradiated area, so that the recovery of absorbance at 500 nm could be observed. Because no absorbance change was observed within 1 h, the rhodopsin molecule could not translate in the gel within the time scale.

For checking a degree of rotational diffusion of the rhodopsin molecule in the gel, light-induced dichroism of the rhodopsin gel was studied by use of the same set-ups as that used in the translational diffusion experiment, except that a polarizer, through which both monitoring and irradiation lights pass, was set in the spectrophotometer. Thus, time courses of absorbance change at 500 nm of the rhodopsin gel were measured with a polarized monitoring light after irradiations of a parallel or a perpendicularly polarized orange light. Because no difference between each time course was observed (data not shown), we concluded that the rhodopsin molecule can rotate in the gel (rotational diffusion was completed within second timescale).

Picosecond absorption measurement and estimation of the percentage of bleaching

An optical setup for the picosecond laser photolysis was described previously (6). To estimate an accurate percentage of bleaching caused by a single picosecond excitation, an experiment was performed as follows.

First, a spectrum of rhodopsin gel was measured with monitoring pulses (wavelength, 470–660 nm; pulse width, 21 ps) from a picosecond laser apparatus at room temperature. This measurement was repeated five times [$A_1(\lambda)$] for increasing a signal-to-noise ratio of the spectrum.

Then the rhodopsin gel was excited with a green pulse (wavelength, 532 nm; pulse width, 21 ps), followed by measurement of a spectrum at 1 ns after the excitation (Fig. 1 *b*). In the measurement, a VO-55 cut-off filter (Toshiba, Tokyo) was placed in front of the polychrometer to exclude scattering light due to the excitation pulse. To avoid an artificial absorption change owing to the excitation with an intense picosecond pulse, we used a weak pulse for the excitation (below 25 μJ/1.8 mmϕ) (7). Then, a difference spectrum before and 1 ns after excitation (wavelength, 550–660 nm) was recorded, which corresponded to a difference spectrum between rhodopsin and bathorhodopsin (6). About 5 min after the excitation (rotational diffusion was completed and no translational diffusion was detected), the spectrum of the rhodopsin gel was measured again with monitoring pulses. This measurement was repeated five times [$A_2(\lambda)$]. Finally, the rhodopsin gel was completely bleached by several tens of times repetition of the excitation with the picosecond green pulses and then the spectrum was measured five times. Because the spectrum coincided with baseline (zero absorbance) in the range from 470 to 660 nm, the percentage of bleaching of rhodopsin by the single excitation was determined according to the following equation:

$$\% \text{ bleaching} = \frac{\int_{470}^{660} A_1(\lambda)\, d\lambda - \int_{470}^{660} A_2(\lambda)\, d\lambda}{\int_{470}^{660} A_1(\lambda)\, d\lambda}. \quad (1)$$

The absolute absorption spectrum of bathorhodopsin at wavelengths longer than 550 nm was calculated from the data thus obtained. The spectrum in the range from 400 to 530 nm was calculated from that previously reported (6). The spectrum of photorhodopsin was calculated from a difference spectrum between the intermediates and rhodopsin measured at 15 ps after excitation, which was previously reported (6). This difference spectrum includes the contribution of bathorhodopsin, because the lifetime of photorhodopsin was estimated to be 45 ps (8). A computer simulation, in which widths of the excitation and the monitoring pulses (25 ps) and the lifetime of photorhodopsin were taken into consideration on the basis of the scheme of primary process of cattle rhodopsin

$$(\text{rhodopsin} \xrightarrow{h\nu} \text{rhodopsin}^* \xrightarrow{\text{fast}} \text{photorhodopsin} \xrightarrow{45\ \text{ps}} \text{bathorhodopsin}),$$

revealed that the difference spectrum measured at 15 ps composed of 67% photorhodopsin and 33% bathorhodopsin. Thus, a real difference spectrum between photorhodopsin and rhodopsin was calculated by subtracting the contribution of bathorhodopsin in the difference spectrum. Then the absolute absorption spectrum of photohodopsin was calculated.

RESULTS AND DISCUSSION

The rhodopsin gel was prepared as described in Materials and Methods. Although an addition of hydroxylamine to the gel is indispensable for estimating the precise percentage of bleaching of rhodopsin, no polymerization of acrylamide took place in the presence of hydroxylamine according to our preliminary experiments. Thus, we first prepared a gel in the sample cell (Fig. 1 *a*) without addition of hydroxylamine. Then hydroxylamine was penetrated into the gel by immersing it into 50 mM hydroxylamine solution (pH 7.0). Fig. 2 *a* shows the absorption spectra of the rhodopsin gel, rhodopsin-digitonin (2%) complex and their completely bleached products (retinal oxime) which were measured through an aperture of 2 mm diameter in front of the sample cell. We confirmed the difference spectrum before and after complete bleaching of the rhodopsin gel was identical to that of rhodopsin in 2% digitonin solution in the presence of 50 mM hydroxylamine (Fig. 2 *b*).

A noteworthy point is that the spectrum of the bleached gel never changed within 1 h (curves 2 and 3 in Fig. 2 *a*), indicating that any translational diffusion of rhodopsin molecule was hampered in the gel. In fact, a colorless hole of 2 mm diameter in red gel could be seen after the experiment. On the other hand, no light-induced dichroism was observed in the gel, indicating that the rapid rotation of rhodopsin molecule took place in the gel.

Fig. 3 *a* shows a difference spectrum between bathorhodopsin and rhodopsin measured at 1 ns after the excitation of rhodopsin gel. This spectrum was almost identical in shape with that measured in 2% digitonin solution (Fig. 3 *b*). The percentage of bleaching of the rhodopsin molecules in the spot of the gel exposed by the pulse was estimated to be 10.9% by calculating Eq. 1 (Fig. 4). This value, however, is not the real percentage of bleaching because the rhodopsin molecules in the gel can rotate three-dimensionally, whereas the absorption spectra measured above gave only two-dimensional information. Thus the real percentage of bleaching was estimated to be 16.4% (10.9 × 3/2).

Using this value, the absolute absorption spectrum of bathorhodopsin was calculated (Fig. 5). Bathorhodopsin at room temperature has a λ_{max} at ~535 nm, which is 8 nm

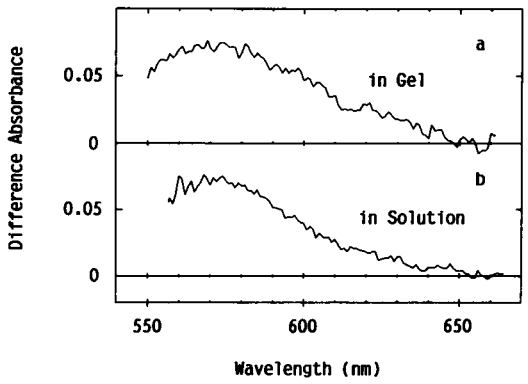

FIGURE 3 Difference spectra between the photoproduct and rhodopsin in rhodopsin gel (*a*) and in 2% digitonin solution (*b*) measured at 1 ns after excitation with a picosecond laser pulse (532 nm, 21 ps). These spectra represent the averages of 17 experiments (*a*) and 54 experiments (*b*), respectively. The excitation pulse energy was 10.4 μJ/1.2 mmϕ.

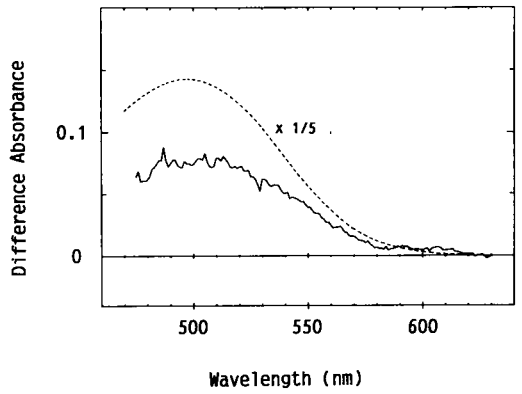

FIGURE 4 Difference absorption spectra of rhodopsins in rhodopsin gel before and after bleaching measured by picosecond laser apparatus. Broken curve shows one fifth of the spectrum of the original rhodopsin (corresponding to the denominator in Eq. 1 and identical to curve 1 in Fig. 2 *a*). Solid curve is a difference spectrum between before and 5 min after excitation with a picosecond laser pulse (corresponding to the numerator in Eq. 1).

shorter than that at low temperature. It should be noted that bathorhodopsin at room temperature has 0.91 times ϵ_{max} than that of rhodopsin, whereas bathorhodopsin measured at low temperature has 1.13 times ϵ_{max} of rhodopsin (9). The spectral difference between room and low temperatures was also reported in case of K intermediate of bacteriorhodopsin (10), in which the spectrum at room temperature has the absorption maximum at 610 nm, 16 nm shorter than that at −190°C, and the extinction

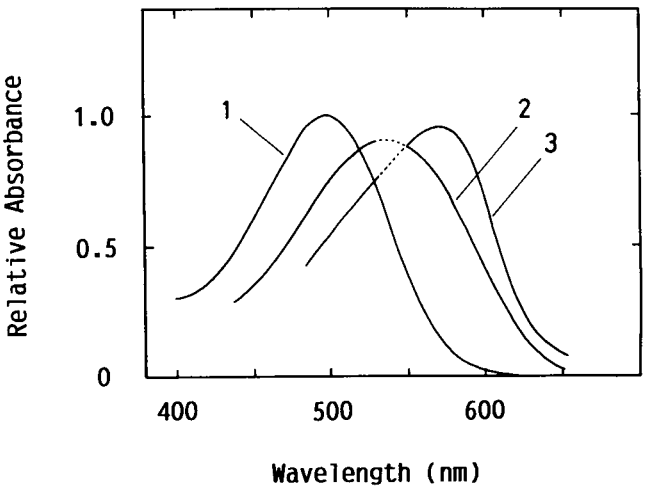

FIGURE 5 Absolute absorption spectra of cattle rhodopsin (curve 1), bathorhodopsin (curve 2), and photorhodopsin (curve 3) at room temperature. Because the scattering of the excitation pulse made it impossible to measure the spectral region from 530 to 550 nm, the spectra in this region were plotted by extrapolating the spectra in other region (*dotted lines*).

coefficient 0.8 times as small as that at −190°C. Therefore, the difference in spectrum between room and low temperatures seems to be common to animal- and bacteriorhodopsin systems.

Another noteworthy point is that the spectral half bandwidth of bathorhodopsin at room temperature (4,660 cm^{-1}; curve 2 in Fig. 5) was broader than those of rhodopsin at room temperature (4,300 cm^{-1}; curve 1 in Fig. 5) and bathorhodopsin at low temperature (4,250 cm^{-1}; [9]). The oscillator strength of bathorhodopsin at room temperature, which can be calculated from its spectrum, was comparable with that of rhodopsin at room temperature, and ~0.9 times that of bathorhodopsin at low temperature. Difference in oscillator strength between bathorhodopsins at room and low temperatures suggests that some difference in potential surface exists between them. Bathorhodopsin at room temperature is rapidly converted to lumirhodopsin through a reaction coordinate, whereas that at low temperature is stable. Therefore, the difference is oscillator strength between them suggests that bathorhodopsin at low temperature would be fallen into some "potential pocket." The energetics and the chromophoric structure of bathorhodopsin at low temperature have been studied by circular dichroism (11, 12), photocalorimetry (13, 14) and resonance Raman spectroscopy (15, 16). As the results, bathorhodopsin has relatively higher energy state than rhodopsin and a chromophore which is in a twisted structure. To compare the states of bathorhodopsins between room and low temperatures, we will research time-resolved measurements at room temperature in the future.

The broader half bandwidth of bathorhodopsin than that of rhodopsin may be explained by an existence of two kinds of bathorhodopsin, which was first reported by low temperature spectrophotometry (17, 18) and then confirmed by nanosecond laser photolysis (19).

Spalink et al. (20) reported the absolute absorption spectrum of bathorhodopsin which was similar to that at low temperature. Their spectrum was calculated on the basis of their result of the relative quantum yield of isorhodopsin to that of rhodopsin (1.1), which was extremely different from those obtained by steady illumination (0.3–0.4) (21–23). Our recent measurements (5) proved that the ratio was 0.39 even under excitation conditions with picosecond laser pulse and became higher when an intense laser pulse was used. Because the high ratio of relative quantum yield they obtained should be attributed to this nonlinear effect caused by an intense laser pulse, the spectrum calculated on the basis of this value should be different from that we obtained.

The absorption spectrum of photorhodopsin has been calculated from the data previously reported (Fig. 5; see Materials and Methods). Photorhodopsin has a λ_{max} at 570 nm, 0.96 times ϵ_{max} of rhodopsin and a half bandwidth

of ~120 nm (4,000 cm^{-1}). Its maximum lay ~35 nm longer than that of bathorhodopsin. Moreover, it should be noted that both spectral bandwidth and oscillator strength were considerably smaller than those of rhodopsin and bathorhodopsin. The small oscillator strength of photorhodopsin is likely to be due to a distortion of the retinylidene chromophore, because an oscillator strength is very sensitive to coplanarity of a conjugated double bond (24), and distortion of a conjugated double bond system from the coplanarity causes a decrease in its value. If so, what bond is distorted? Our recent results of picosecond laser photolysis of rhodopsin analogues having C_{11}-C_{12} locked retinal analogues revealed that the chromophore of photorhodopsin should be in an all-*trans* form at C_{11}-C_{12} double bond with nearby single bonds highly distorted (25). Therefore, photorhodopsin is likely to be an intermediate state in isomerization of the chromophore.

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