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FORMATION OF HYPSORHODOPSIN AT ROOM TEMPERATURE BY PICOSECOND GREEN PULSE

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Excitation of squid rhodopsin with a single laser pulse (532 nm, 25 ps) at 18°C yielded photorhodopsin, a precursor of bathorhodopsin. In the linear region, no relation between amount of photorhodopsin and excitation-energy hypsorhodopsin was detected, while in a photon saturation region this was observed. The time constant of hypsorhodopsin to bathorhodopsin decay was about 125 ps. Dependencies of formation of photorhodopsin and hypsorhodopsin on the excitation energy suggest that hypsorhodopsins of squid and octopus are formed by a two-photon reaction. No cattle hypsorhodopsin was detected in our experimental conditions.

Introduction

The initial photochemical process of visual pigment has been studied by low temperature spectrophotometry [1–6]. When rhodopsin was irradiated with yellow light at liquid helium temperature, hypsorhodopsin was produced, whereas the irradiation with blue light yielded bathorhodopsin. Thus, the question arises as to which intermediate is the first photoproduct. One of the most effective means to solve this question has been thought to be an application of picosecond laser photolysis. Shichida et al. [7] observed squid hypsorhodopsin as a precursor of bathorhodopsin on excitation of rhodopsin with a laser pulse at 347 nm. In contrast, most experiments [8–10] on cattle rhodopsin excited with 530 nm laser pulse (pulse width, 6 or 7 ps) failed to detect hypsorhodopsin except for that mentioned by Kobayashi [11], who carried out the experiment under similar conditions. In this way, there is still disagreement in formation of hypsorhodopsin among research groups.

On the other hand, Peters et al. [9] reported the presence of another precursor of cattle bathorhodopsin, which they considered as an excited state of rhodopsin, from the laser photolysis of cattle rhodopsin at liquid helium temperature. Later, its precursor was called 'batho" by Honig et al. [12]. Though this product was observed below 20 K, they failed to confirm its formation at room temperature. Shichida et al. [7] suggested the presence of a precursor of squid hypsorhodopsin from the kinetics of formation of hypsorhodopsin by 347 nm excitation of rhodopsin at liquid nitrogen temperatures. Very recently, we have confirmed the presence of a bathochromic photoproduct which is produced earlier than bathorhodopsin at room temperature. This photoproduct was produced by excitation of cattle, squid and octopus rhodopsins with relatively less intense pulse than those used by Peters et al. [9] and Kobayashi [11]. The time constant of the decay of this product to bathorhodopsin was about 40 ps in the case of the

* To whom correspondence should be addressed. Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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cattle rhodopsin system and several hundreds of picoseconds in the case of squid and octopus rhodopsins. We called this new photoproduct 'photorhodopsin' [13].

The questions that confront us are whether or not excitation wavelength and animal specificities are present in the process of formation of hypsorhodopsin, and in what way hypsorhodopsin is produced from rhodopsin. The present paper gives the results of the picosecond kinetics by 532 nm excitation of squid, octopus and cattle rhodopsins, suggesting that hypsorhodopsin may be produced by a two-photon reaction.

Materials and Methods

Preparation of squid rhodopsin. The preparation of squid (*Todarodes pacificus*) rhodopsin was carried out under dim red light according to a method described by Yoshizawa and Shichida [6]. The microvilli containing rhodopsin were isolated from the retinal homogenate by a sucrose linear gradient (25–45% (w/v) in the 10 mM Hepes buffer, pH 7.0). Rhodopsin was extracted from the microvilli with 2% digitonin dissolved in 10 mM Hepes buffer (pH 7.0). In the extract no retinochrome was detected because addition of neutralized hydroxylamine (final concentration: 100 mM) to the extract caused no spectral change in the visible region. After concentration the rhodopsin extract by ultrafiltration membranes (Amicon), it was mixed with glycerol to give a final concentration of 66%. The absorbance of rhodopsin at 532 nm in an optical cell (light pathlength: 2 mm) was adjusted to 0.7–0.8.

Preparation of octopus rhodopsin. Octopus (*Octopus ocellatus*) rhodopsin was extracted by a method similar to that of squid rhodopsin. Glycerol was added to the extract to give a final concentration of 50%. The absorbance of the extract at 532 nm in the optical cell was about 0.5.

Preparation of cattle rhodopsin. Rod outer segments were prepared by a conventional sucrose flotation method [14–16]. Cattle rhodopsin was extracted from rod outer segments with 2% digitonin dissolved in 10 mM Hepes buffer (pH 7.0). No glycerol was added to the extract. The absorbance at 532 nm in the optical cell was adjusted to about 1.0.

The optical system for picosecond kinetics measurement The optical system is a double beam picosecond spectrometer, which is basically similar to that described by Shichida et al. [17]. A train composed of picosecond fundamental pulses (1064 nm) was generated by a mode-locked Nd^{3+} : YAG laser (Matsui). A single pulse was isolated from the pulse train and then amplified to about 30 mJ by two sets of amplifiers. The pulse was focused on a phase-matched K^2H_2P crystal for generating the second harmonic (532 nm). The 532 nm pulse thus obtained was focused on the center of an optical cell containing ^2H_2O or a mixture of ^2H_2O and H_3PO_4 to generate a monitoring pulse composed of several wavelengths. The wavelength of the pulse was selected by an interference filter. The fundamental pulse from the K^2H_2P crystal was again focused on another phase-matched K^2H_2P crystal for generating the 532 nm pulse, which was focused on a sample cell after passing through a movable (delay) prism. After being focused on a diffuser (ground glass), the monitoring pulse was collimated on an echelon reflector, then split into two beams by a half mirror; one was used for monitoring the absorption change of the sample in the optical cell and the other for correcting the intensity of the monitoring pulse. The monitoring pulses were focused on a slit of a polychrometer (Jarrell Ash) and detected by a Vidicon detector (ISIT, PAR).

For simultaneous measurements of kinetics at two wavelengths, the monitoring beam consisting of two wavelengths was focused on the two-dimensional detector after being reflected by the echelon. During the expeirment, the excitation energy of the pulse was monitored by a biplanar photodiode (Hamamatsu). At the end of the experiment, the energy was calibrated by a joulemeter (GENTEC) placed in front of the optical cell containing the sample. The sample in the optical cell was renewed at every excitation with the pulse. All experiments were performed at 18°C.

Results

Squid rhodopsin was excited with a single 532 nm laser pulse. The time-resolved absorbance changes at 558 and 420 nm were measured simultaneously. As shown in Fig. 1, no increase of

absorbance at 420 nm was observed, indicating no formation of hypsorhodopsin, while the absorbance at 558 nm increased remarkably owing to formation of photorhodopsin and bathorhodopsin. The absorbance change of photorhodopsin to bathorhodopsin is not visible because both intermediates are roughly equal in absorbance near 558 nm [13]. Presumably, photorhodopsin is dominant in early stage, and in late stage bathorhodopsin is dominant.

In Fig. 2, an intense laser pulse was used for excitation of the rhodopsin. The absorbance at 420 nm increased within 15 ps due to formation of hypsorhodopsin, and then gradually decreased with a time constant of about 125 ps. The absorbance at 558 nm also increased within 15 ps indicating that photorhodopsin was formed within 15 ps, but it continued to increase gradually with a time constant of about 125 ps, which agrees with that of absorbance decrease at 420 nm. In Fig. 2, the change at 558 nm from 69 to 555 ps is 2.1-times larger in absorbance than that at 420 nm. This value is in good agreement with that obtained by low temperature spectrophotometry (2.0, (3)). Therefore, hypsorhodopsin decayed to bathorhodopsin at room temperature as well as at low temperature.

A relationship between the excitation energy and absorbance change at 578 or 420 nm is shown in Fig. 3a. These absorbance changes were measured at 15 ps after 532 nm excitation. This time interval was adopted for preventing contamination with absorbance change due to the thermal decay of hypsorhodopsin to bathorhodopsin (τ, 125 ps). The change at 578 nm linearly increased in the range of excitation energy from 0 to about 25 μJ/1.8 mm ∅. Above 100 μJ/1.8 mm ∅, it reached a constant level. On the other hand, the change at 420 nm was not observed below 25 μJ/1.8 mm ∅. Above 100 μJ/1.8 mm ∅, it continued to increase. Since the rise of absorbance at 420 and 578 nm at 15 ps after the excitation is mainly due to formation of hypsorhodopsin and photorhodopsin, respectively, this result means that hypsorhodopsin is formed in the high range of excitation energy where formation of photorhodopsin saturates. Thus, it will be inferred that hypsorhodopsin may be formed by a multiphoton

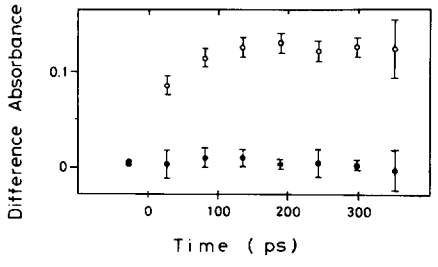

Fig. 1. Absorbance changes at 558 (○) and at 420 nm (●) after excitation of squid rhodopsin with a laser pulse (excitation energy, approx. 20 μJ/1.8 mm ∅; wavelength, 532 nm; pulse width, 25 ps). Each point is the average of 15 experiments.

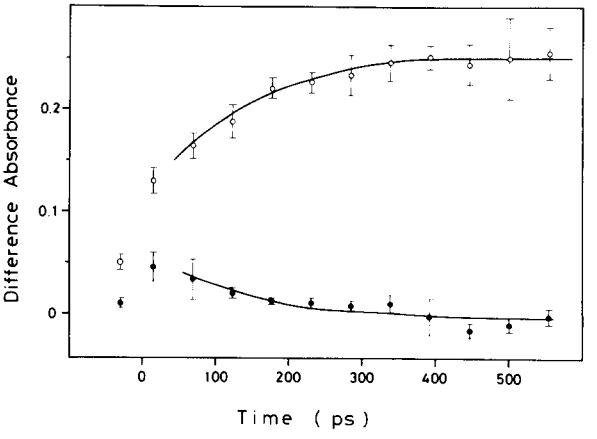

Fig. 2. Absorbance changes at 558 (○) and at 420 nm (●) after excitation of squid rhodopsin with an intense laser pulse (excitation energy, approx. 120 μJ/1.8 mm ∅; wavelength, 532 nm; pulse width, 25 ps). Each point is the average of 15 experiments.

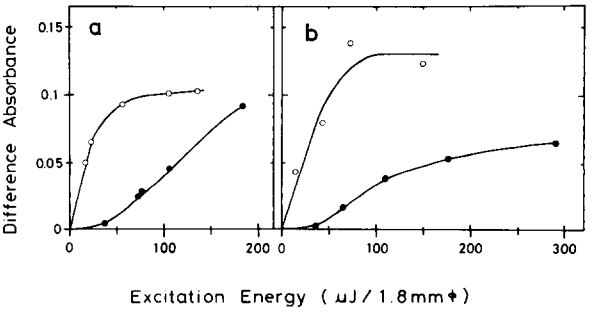

Fig. 3. Excitation-energy dependency of absorbance changes at 578 (○) and at 420 nm (●) after excitation of squid (a) and octopus (b) rhodopsins with a 532 nm pulse. The absorbance changes were measured at 15 ps after the excitation. Each point is average of 5–15 experiments.

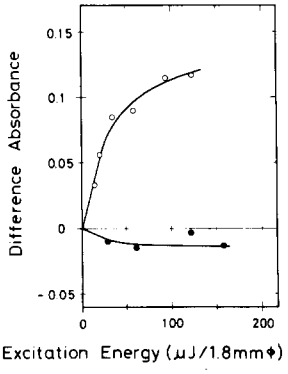

Fig. 4. Excitation-energy dependency of absorbance changes at 578 (○) and at 420 nm (●) after excitation of cattle rhodopsin with a 532 nm pulse. The absorbance changes were measured at 15 ps after the excitation. Each point is the average of 5–15 experiments.

reaction, while photorhodopsin is formed by a single photon reaction.

Similar experiments have been carried out in octopus rhodopsin as shown in Fig. 3b. The absorbance change at 578 nm displayed a linear relation to the excitation energy below 50 μJ/1.8 mm ∅. The absorbance change at 420 nm was relatively small in this energy range. Above 100 μJ/1.8 mm ∅, the absorbance at 578 nm began to saturate, where the absorbance change at 420 nm continued to increase. These results were fundamentally similar to those of squid rhodopsin.

In case of cattle rhodopsin, no increase of absorbance at 420 nm was observed even by the laser pulse at 160 μJ/1.8 mm ∅ (Fig. 4). On the other hand, the absorbance at 578 nm increased as the excitation energy increased. This increase may be due to formation of photorhodopsin. Below 30 μJ/1.8 mm ∅, absorbance change was proportional to the excitation energy.

Discussion

These experimental results described above were obtained by use of detergent extracted rhodopsin in 10 mM Hepes buffer and 66% glycerol as a sample which is not the same in condition as that in the retina. However, since Horiuchi et al. [18] reported that hypsorhodopsin was produced from rhodopsin also in the frog retina, as well as that extracted by detergent, our expeirmental results seems to be applicable to those in the retina.

The present experiments clearly show that hypsorhodopsin can be produced at room temperature only when squid and octopus rhodopsins are excited with the intesne laser pulse, though no cattle hypsorhodopsin is detected. The possible mechanisms of formation of hypsorhodopsin may be as follows: (1) two-photon absorption by rhodopsin; (2) one-photon absorption by the singlet excited state of rhodopsin; (3) one-photon absorption by photorhodopsin; (4) one-photon absorption by bathorhodopsin.

The formation of squid or octopus hypsorhodopsin was easily observed by irradiation with continuous yellow light (larger than 480 nm) at liquid helium temperature (4 K) [3–5]. Under this light condition, it is highly improbable that two-photon absorption by a single rhodopsin molecule would occur. Assuming that the mechanism by which hypsorhodopsin is formed at room temperature is applicable to the formation of hypsorhodopsin at liquid helium temperature, the second possibility can be also ruled out, because the life time of the singlet excited state at 4 K [9] is too short to absorb the second photon, which leads to formation of hypsorhodopsin at 4 K by continuous irradiation. The fourth possibility was also ruled out because bathorhodopsin did not appear within 25 ps (excitation pulse width) [13]. Thus the third mechanism may be possible.

On the basis of the discussion mentioned above, the schemes for the initial photochemical process of rhodopsin at room temperature are shown in Fig. 5. In case of cattle rhodopsin, the photon

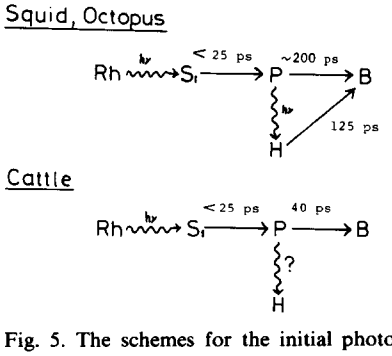

Fig. 5. The schemes for the initial photochemical process of rhodopsin at room temperature. Rh, rhodopsin; S_1, singlet-excited state; P, photorhodopsin; H, hypsorhodopsin; B, bathorhodopsin.

saturation effect took place in the energy range above 20 μJ/1.8 mm ∅ (7.9 J/m^2), where formation of hypsorhodopsin could not be observed at least below 160 μJ/1.8 mm ∅ (62.9 J/m^2) (Fig. 4). In most publications in this field, there is no description of the excitation-energy density. Monger et al. [10] reported that excitation of cattle rhodopsin at energy density of 60 J/m^2 (pulse width, 7 ps) induced to an increase of absorbance near 460 nm persisting for at least 100 ps, but they did not assign this photoproduct to hypsorhodopsin for the following reason. Since rise of absorbance at 460 nm was in parallel with the formation of bathorhodopsin, they thought that the 460 nm photoproduct should not be hypsorhodopsin on the basis of the sequential conversion of hypsorhodopsin to bathorhodopsin, as was observed by Shichida et al. [7]. However, it could be possible to assign the photoproduct to hypsorhodopsin if Kobayashi's description [11] was taken into consideration, in which the formation of hypsorhodopsin was observed under the similar condition to that of Monger et al. As already stated, the present experiment failed to demonstrate the formation of cattle hypsorhodopsin at room temperature. This is probably owing to the small excitation-energy denisty; Monger et al. and Kobayashi used 32-times larger energy density per unit time than that we have used for measurement of the absorbance change in a linear region. The highest energy which we have used is only 8-times larger than that in a linear region. It may be possible to observe the formation of hypsorhodopsin if we use more intense pulse.

Nanosecond flash photolytic experiments of cattle rhodopsin displayed formation of a photoproduct having a peak near 440 nm at 50 ns after the excitation [19]. Since this photoproduct is similar in decay constant to bathorhodopsin (145 ± 25 ns) [19], it cannot be identified as hypsorhodopsin which decays with time constant of 125 ps. The assignment of the photoproduct remains unsolved in the present experiment.

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