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TRANSIENT SPECTRA OF INTERMEDIATES IN THE PHOTOLYTIC SEQUENCE OF OCTOPUS RHODOPSIN

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Summary

The intermediate photolytic sequence of octopus rhodopsin was studied at different temperatures and different pH values by means of a flash photolysis-rapid scan spectrophotometry near physiological temperature.

The first photoproduct in the photolysis of rhodopsin was lumirhodopsin. Transformation of lumirhodopsin → mesorhodopsin took place independently of the pH of the solution. Mesorhodopsin was transformed to acid metarhodopsin in acid solution. In alkaline solution, mesorhodopsin was transformed to transient acid metarhodospsin whose absorption spectrum was similar to acid metarhodopsin. Transient acid metarhodopsin was then transformed to alkaline metarhodopsin reaching a tautomeric equilibrium which was determined by the pH of the solution.

Introduction

When rhodopsin is illuminated in vitro there is an initial photoisomerization of the 11-*cis* retinal to all-*trans* form, followed by a series of intermediates to the final photoproduct [1]. One or more of these thermal reactions, meeting the appropriate temporal requirement, might conceivably lead to visual excitation. Thus, it is important to identify the sequence of intermediates in the photochemical cycle of rhodopsin.

The final photoproduct of invertebrate rhodopsin is metarhodopsin which is stable at physiological temperatures; rhodopsin can be photochemically regenerated from metarhodopsin. The sequence of intermediates in the photochemical cycle of rhodopsin has often been studied using invertebrates [2—7]. Octopus rhodopsin, which was used in the present work, is an excellent material for the study of the photochemical cycle (rhodopsin → metarhodopsin

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and metarhodopsin → rhodopsin) because the absorption maximum of acid metarhodopsin (512 nm) is located at a much longer wavelength than that of rhodopsin (476 nm) (in *Paroctopus defleini*). Consequently, the interconversion of rhodopsin and metarhodopsin can be easily induced by alternate irradiation with blue and red light.

The life time of the intermediates appearing in the photolysis of rhodopsin is very short at physiological temperature [8] and atmospheric pressure, so the spectra of the intermediates were obtained previously at either cryogenic temperatures [9,10] or under high pressure [11].

This paper describes some of the results for the photolytic sequence of octopus rhodopsin at near physiological condition which were studied by means of a flash photolysis-rapid scan spectrophotometric technique that permits the simultaneous measurement of transient absorption spectra of the intermediate immediately after a single flash.

Material and Methods

Extraction and purification of rhodopsin

Octopuses (Mizudako, *Paroctopus defleini defleini*) were caught at Oshidomari on the Rishiri island, in northern Hokkaido, Japan. They were collected and decapitated at once in dim light, and their eyeballs, kept in the dark at −20°C, were brought to the laboratory and stored in a freezer.

Octopus rhodopsin was prepared by the methods similar to those described elsewhere [4]. The outer segments of the photoreceptor cells were isolated from bisected eyes by shaking in 10 mM imidazole-HCl buffer (pH 7.2) containing 1 mM $MgCl_2$ and 500 mM NaCl and microvillar membranes were isolated by repeated sucrose floatations (40%, w/v). The preparation, however, contained membranes other than microvillar membrane. To obtain the pure microvillar membranes, the following procedures were performed. The membranes were suspended in acid solution (10 mM imidazole-HCl buffer (pH 5.7)) and sedimented at 25 000 × *g* for 30 min. The pellet was washed twice with distilled water. Only the loose pellet was collected. Then, the microvillar membranes were further purified by discontinuous sucrose gradient centrifugation. The membranes suspended in sucrose buffer (density of 1.20 g/ml) were layered at the bottom of a discontinuous sucrose gradient in 10 mM imidazole-HCl (pH 7.2) containing 1 mM $MgCl_2$ and 500 mM NaCl. The gradient was formed by layering sucrose solutions of different densities (1.15, 1.13 and 1.11 g/ml). After centrifugation at 21 000 × *g* for 2 h at 5°C, the orange band appearing at the 1.13/1.15 g/ml interface was collected using a syringe with a long needle (No. 18). The dense orange band at the 1.15/1.17 g/ml interface contained microvillar membranes, but it also contained the nerve membrane fragments. If we avoided suspending the membrane in an acid buffer (10 mM imidazole buffer, pH 5.7), only a small amount of the microvillar membrane appeared at the 1.13/1.15 g/ml interface. The isolated microvillar membranes were washed repeatedly with distilled water, 100 mM imidazole, 100 mM imidazole-HCl (pH 7.2), and finally, with 0.01% digitonin solution. Rhodopsin was extracted from the microvillar membranes with 2% digitonin in 10 mM imidazole-HCl buffer (pH 7.2) at 4°C for 2 h. The extracts were then cleared by centrifugation (25 000 × *g*, 60 min). The rhodopsin extracts were

purified on a DEAE-cellulose column after the method of Suzuki et al. [3]. The pH of the rhodopsin solution was adjusted such that the buffer in the solution was repeatedly exchanged with 100 mM imidazole-HCl buffer at an appropriate pH containing 1% digitonin using an Amicon Diaflow membrane ultrafilter Type CF25. The solution contained only digitonin and buffer, no glycerol, even though the sample temperature was −1.6°C.

Methods

The optical absorption spectra were taken with a recording spectrophotometer (Hitachi model 200). Actinic light was obtained from a 500 W Xe-short arc lamp (Ushio) which had passed through glass filters (Toshiba VV42 or VY52) transmitting the required wavelength. This light was focused by an optical system and led to the sample cell through an optical fibre (5 mm in diameter).

Transient absorption spectra were observed using a rapid-scan spectrophotometer (UNION GIKEN RA-1300) equipped with a flash apparatus. The details of the apparatus were described elsewhere [4,12]. An image of panchromatic light from a halogen lamp (Osram 50 W) fed by a well-stabilized source entered a sample cell and the light was dispersed by a grating and received by an image decector tube (HTV-R571). The spectral pattern can be measured in a wavelength span of 150 nm with 11 steps of scan speed from 1 ms to 10 s. In the present experiments, the scan speed was fixed at 30 nm/ms, that is, the wavelength span of 150 nm was scanned in 5 ms. The scanning of the spectrum was started at an appropriate time after the flash and the scans were recorded by a transient memory. Up to five separate spectra can be recorded. Each transient signal is digitized with an 8-bit (256 data unit) resolution and stored in a 9-bit (512 channel) memory. An analog replica could be recorded on an X-Y recorder as a spectral pattern of the absorbance.

In general the monitoring source intensity was set sufficiently low to avoid sample bleaching. However, the sample absorbance was changed by the monitoring light if the shutter was open for more than approx. 1 s. In observations lasting over 1 s, the shutter was opened only when the absorption spectrum was recorded.

The flash lamp had a half duration of about 200 μs. The beam was focused on the sample cell using an elliptical mirror, a thermal filter and coloured glass filters.

Measurements were made at temperatures of −1.6°C and 6.8°C in a double jacketed cylindrical cuvette. The outer jacket which contained its own windows was evacuated to prevent fogging, and cold ethanol from a low temperature bath (Lauda model) was circulated in the inner jacket. The temperature within the sample cell was measured with a thermocouple.

Results

Absorbance spectra of octopus visual pigments

Absorbance spectra of rhodopsin, alkaline metarhodopsin and acid metarhodopsin of octopus were determined at 12°C. Fig. 1 shows the spectral changes in the interconversion of rhodopsin and metarhodopsins. Wehn

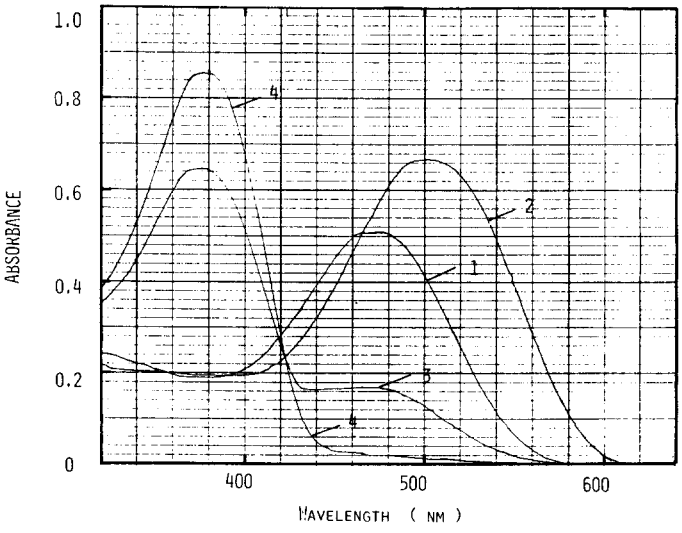

Fig. 1. Absorbance spectra of octopus rhodopsin. Rhodopsin at pH 5.6 (curve 1) was irradiated with blue light (λ = 420 nm) for 5 min (curve 2) and then the pH was raised to 10.5 by addition of a small amount of saturated Na_2CO_3 (curve 3). Finally, the solution was irradiated with yellow light ($\lambda > 520$ nm) for 5 min and all the rhodopsin was converted alkaline metarhodopsin. All spectra were measured at 12°C.

octopus rhodopsin was irradiated with blue light (Toshiba VV42) for 5 min at pH 5.6, a steady-state mixture of rhodopsin and acid metarhodopsin (curve 2) was formed. Then the solution was brought to pH 10.5, by the addition of small amount of saturated Na_2CO_3, in order to convert acid metarhodopsin to alkaline metarhodopsin (curve 3). All the rhodopsin remaining was then converted to alkaline metarhodopsin by further irradiation with yellow light ($\lambda > 520$ nm), as shown by curve 4.

Transient absorbance changes in the photoconversion of rhodopsin to metarhodopsin

Fig. 2A shows the results of transient spectral changes in the photolysis of octopus rhodopsin at −1.6°C observed by the rapid-scan spectrophotometer equipped with a flash apparatus (scan speed constant at 30 nm/ms). Curve 1 in Fig. 2A shows the absorption spectrum of the rhodopsin solution containing 2% digitonin and 100 mM imidazole-HCl buffer (pH 5.6) before flashing. Immediately after the flash with blue light (Toshiba VV42), there was an initial abrupt change in the absorption spectrum within the duration of the flash and time response of the system of the apparatus. By the time curve 2 in Fig. 2A was recorded, the first photoproduct in the present experiment was already formed; its spectrum is at longer wavelengths and it has a higher peak absorption than the original rhodopsin.

During the next 50 ms, the spectrum shifted to the longer wavelengths and increased in absorbance. Note that while there is appreciable gain in absorbance up to 470 nm, there is littel, if any, increase to the blue of this wavelength. This shift represents the first dark reaction (Process I) which could be recorded. Similar transient spectral changes in the photolysis of rhodopsin with blue light

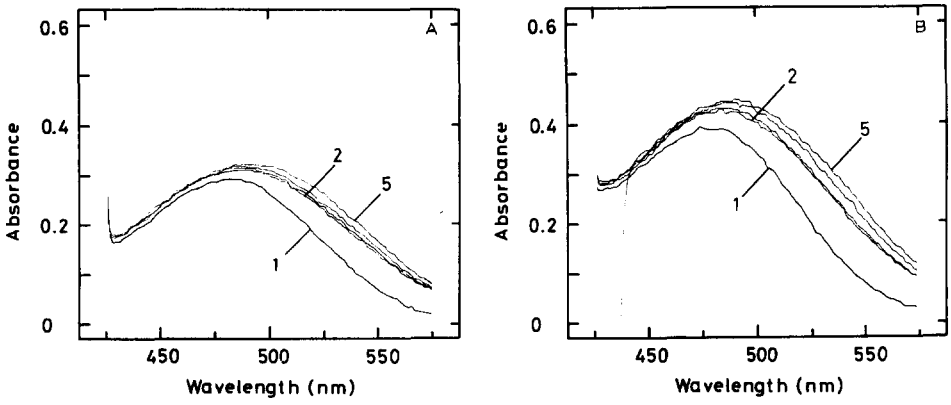

Fig. 2. Transient absorbance spectra in the photolysis of octopus rhodopsin at —1.6°C. The pH of the solution was (A) 5.6 and (B) 10.2. Scanning speed was 30 nm/ms. Curve 1 in (A) and (B) is the absorbance spectrum of rhodopsin before the flashing. The scanning of spectra 2, 3, 4 and 5 were started at (A) 0 ms, 5 ms, 20 ms, 50 ms, (B) 0 ms, 15 ms, 50 ms, 100 ms, after a blue light flash.

irradiation (Fig. 2B) were also observed for an alkaline solution (pH 10.2) at the same temperature.

During the next 500 ms, the spectrum began to shift toward the red and the curves passed through an isosbestic point at approx. 480 nm with a concomitant increase in absorbance around at 520 nm and a decrease in absorbance at 460 nm as shown in Fig. 3A (Process II).

Transient spectra in the photolysis of rhodopsin were also observed for the alkaline solution (pH 10.2) at the same temperature. Figs. 2B and 3B show the transient spectral changes from the time immediately after the flash to 100 ms and 50 ms to 200 ms, respectively. Transient spectra shown in Figs. 3A and 3B were very similar to each other though the sample pH values in solution were

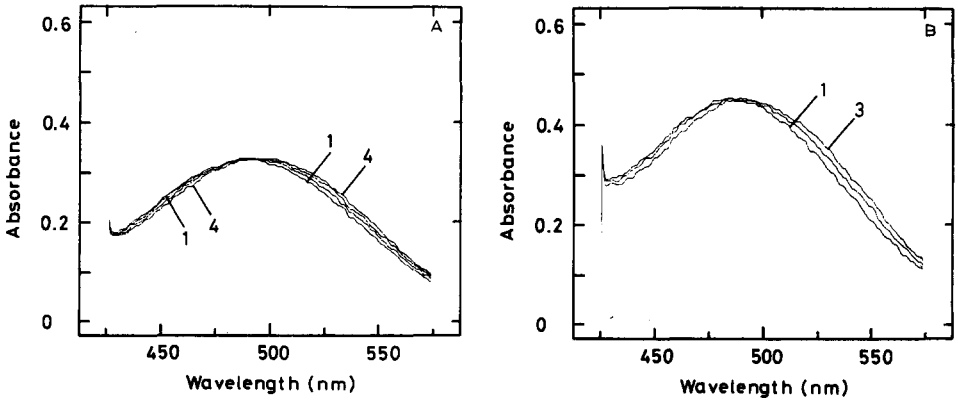

Fig. 3. Transient absorbance spectra in the photolysis of octopus rhodopsin at —1.6°C. The pH of the solution was (A) 5.6 and (B) 10.2. Scanning speed was 30 nm/ms. The scanning of the spectra 1,2, 3 and 4 in (A) were started at 50 ms, 100 ms, 200 ms and 800 ms and that of spectra 1, 2 and 3 in (B) were at 50 ms, 100 ms, 200 ms after a blue light flash.

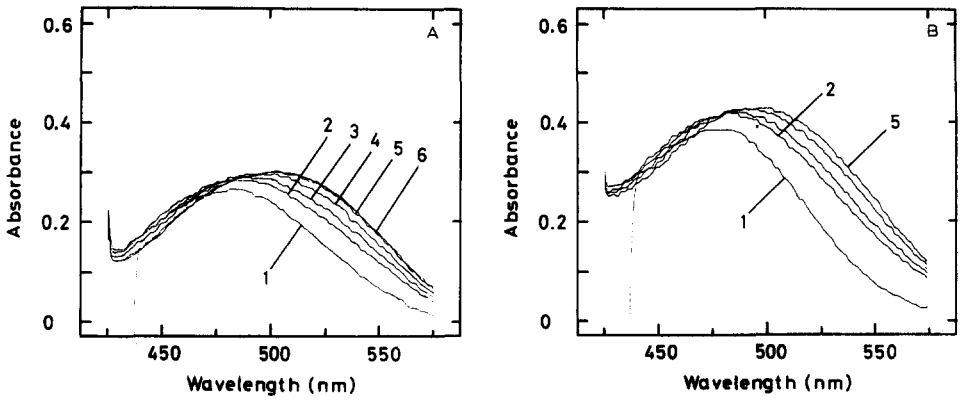

Fig. 4. Transiwnt absorbance spectra in the photolysis of octopus rhodopsin at 6.8°C. The pH of the solution was (A) 5.6 and (B) 10.2. Scanning speed was 30 nm/ms. Curve 1 in (A) and (B) is the absorband spectrum of rhodopsin before flashing. The scanning of spectra 2, 3, 4, 5 and 6 in (A) were started at 0 ms, 5 ms, 25 ms, 100 ms, 120 s and that of spectra 2, 3, 4 and 5 in (B) were at 0 ms, 5 ms, 25 ms, 50 ms after a blue light flash.

quite different. This shows that Process II as well as Process I was insensitive to pH from 5.6 to 10.2.

The sample temperature was then raised to 6.8°C and the transient spectral changes in the photolysis of rhodopsin were observed in acid solution (pH 5.6) and in alkaline solution (pH 10.2) as shown in Figs. 4A and 4B, respectively. Curve 1 in Fig. 4A, which was recorded immediately after a flash, was similar to curve 1 in Fig. 3A recorded at 100 ms. The pattern of spectral changes in Fig. 3A is very similar to that in Fig. 4A, though their time scale is quite different. The same thing could also be said for Figs. 3B and 4B in alkaline solution. Two transformations, Process I and Process II, were observed at −1.6°C. However, only one transformation, Process II, could be observed at 6.3°C due to the time resolution of the present measuring system. These results show that the rate of transformation of both Processes I and II increase with increasing temperature.

The absorption spectra at 100 ms, curve 5 in Fig. 4A, and at 50 ms, curve 5 in Fig. 4B, were very similar though their pH was quite different. During the next 120 s, transient spectral curves in alkaline solution were quite different from those in acid solution. Figs. 5A and 5B show transient absorption spectra of the alkaline solution at 6.8°C from 100 ms to 120 s at the near ultraviolet and visible wavelength region, respectively. The shape of the spectrum at 0.1 s was quite different from that at 120 s after the flash. In the figures, the absorption band around 500 nm fell and the band around 380 nm appeared with an isosbestic point at approx. 425 nm (Process III).

On the other hand, the spectrum of the acid solution still is unchanged after 100 ms, that is, there was no significant difference between spectrum at 100 ms after the flash (curve 5 in Fig. 4A) and that at 120 s (curve 6 in Fig. 4A) at 6.8°C.

The final photoproduct of octopus rhodopsin in acid solution is acid metarhodopsin. Fig. 4A shows that acid metarhodopsin was formed at 100 ms after

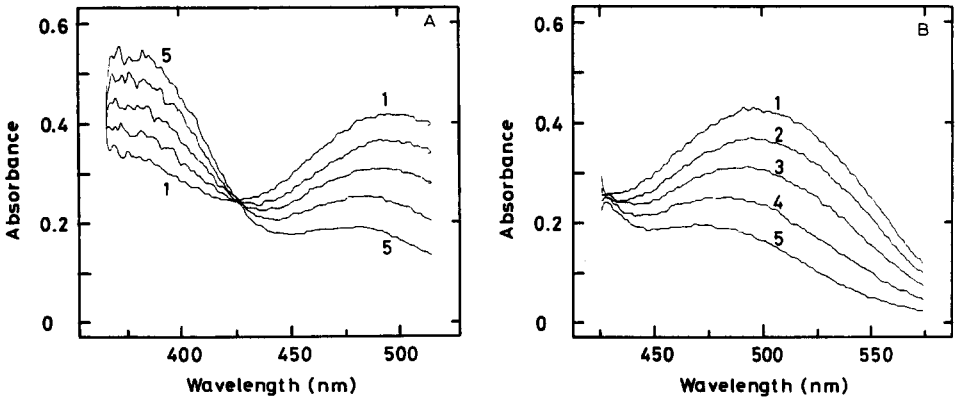

Fig. 5. Transient absorbance spectra in the photolysis of octopus rhodopsin at pH 10.2 and 6.8°C. Scanning speed was 30 nm/ms. The scanning of spectra 1, 2, 3 and 4 in (A) and (B) were started at 0.1 s, 0.4 s, 0.8 s, 1.6 s and 120 s after a blue light flash.

the flash at 6.8°C. Transient absorption spectrum appeared at 50 ms (curve 5 in Fig. 4B) in alkaline solution is very similar to that of acid metarhodopsin. This intermediate is denoted as transient acid metarhodopsin (t-acid metarhodopsin). Figs. 5A and B show that t-acid metarhodopsin is converted to alkaline metarhodopsin which is the final photoproduct of octopus rhodopsin in alkaline solution.

Discussion

We observed that the transient absorption spectra of the intermediate appeared in Processes I, II and III. However, the intermediate process and the spectrum of the intermediate in the photolytic sequences of octopus rhodopsin have never been reported. The photolytic sequence of squid (*Todarhodes pacificus*) has been extensively studied by the method of flash photometry [13,14], low temperature spectrophotometry [9,10] and spectroscopy under high pressure [11]. The results may help to identify the spectrum of intermediates of octopus visual pigments. Process III which is shown in Figs. 4A and B was also reported by Ebina et al. [14] for squid (*Todarhodes pacificus*) and octopus (Amadako) by the method of flash photometry. They concluded that this transformation in alkaline solution is that of acid metarhodopsin to alkaline metarhodopsin. However there is no evidence without spectral similarity that 'acid metarhodopsin' in alkaline solution is the same pigment as acid metarhodopsin in acid solution.

Our recent work [15] showed that the temperature and pressure effects on the rate of formation of acid metarhodopsin in acid solution and 'acid metarhodopsin' in alkaline metarhodopsin were quite different. Accordingly, the precursor of alkaline metarhodopsin is denoted as t-acid metarhodopsin.

Ebina et al. [13,14] showed that a precursor of acid metarhodopsin of squid is mesorhodopsin. In the transformation of mesorhodopsin to acid metarhodopsin, it was reported that absorbance at wavelengths shorter than 455 nm decreased and absorbance at around 500 nm increased with isosbestic points at

455 nm and 545 nm, independent of the pH of the solution. On the other hand, the wavelength of the isosbestic point of the spectra shown in Figs. 4A and 4B was at around 480 nm. Though the wavelength of the isosbestic point in Process II of octopus was differen from that in the transformation of mesorhodopsin to acid metarhodopsin of squid pigments, the trend of the spectral change in these two processes closely resembles to each other. Accordingly, Process II seems to be the transformation of mesorhodopsin to acid metarhodopsin. Tokunaga et al. [10] found intermediate LM as a precursor of acid metarhodopsin in the photoconversion of squid rhodopsin in cryogenic temperature. Though the absorption maximum of intermediary LM is compatible with that of mesorhodopsin, intermediate LM has a higher extinction coefficient than acid metarhodopsin. Suzuki et al. [6] found that the formation of *P*-465 in the temperature range between −80°C and −15°C, and an absorption maximum located at 465 nm; the extinction was 1.22 times higher than that of rhodopsin. Accordingly, intermediate LM may be contaminated with *P*-465 as it was found at cryogenetic temperatures. Our present results for octopus rhodopsin support the results given by Ebina et al. [14], that is, mesorhodopsin has a lower extinction coefficient than acid metarhodopsin, for either squid or octopus. As the configuration of the chromophore in *P*-465 was shown to be neither all-*trans* retinal nor 11-*cis* retinal, it may be that it should not be included in a photolytic sequence of rhodopsin at physiological conditions.

Assuming the photolytic sequence of squid rhodopsin is valid as for that of octopus rhodopsin; Process I, which was the first dark reaction observed in the present experiments, may be considered to be the transformation of lumirhodopsin to mesorhodopsin. However, spectral changes in Process I of octopus shown in Fig. 2A and Fig. 2B were different from those in the transformation of lumirhodopsin to mesorhodopsin of squid where the absorbance at a shorter wavelength region of an isobestic point (520 nm) arose and that at longer wavelength fell. This discrepancy may arise from the following reasons; (1) due to the difference in spectrum of intermediate of octopus and squid. (2) The observation of squid visual pigments was studied at cryogenic temperature, and the preparation contained glycerol. Curve 1 in Fig. 2A and Fig. 2B is the first observation of cephalopod lumirhodopsin at around physiological temperature.

The photochemical sequence of octopus rhodopsin observed in the present experiment can be illustrated as follows

$$\begin{array}{l} \text{Rhodopsin} \xrightarrow{h\nu} \text{lumirhodopsin} \rightarrow \text{mesorhodopsin} \rightarrow \text{acid metarhodopsin} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \overset{H^-\downarrow\uparrow H^+}{} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \text{T-acid metarhodopsin} \rightarrow \text{alkaline metarhodopsin} \end{array}$$

In the present experiment the first photoproduct in the photolysis of octopus rhodopsin was lumirhodopsin. However, the abrupt change in the absorbance from rhodopsin to lumirhodopsin suggests that there may exist a precursor of lumirhodopsin. This precursor may be a pigment like bathorhodopsin since it is well known that the precursor of lumirhodopsin in other cephalopods is bathorhodopsin [10]. As the formation process of forming lumirhodopsin could not be clarified because of the experimental limitations, the first transformation studied in the present work was that of lumirhodopsin to mesorhodopsin. This transformation could be observed either under acid or

alkaline conditions, though the rate of transofrmation seemed to be affected by the pH.

In the transformations of mesorhodopsin to acid metarhodopsin in acid solution and mesorhodopsin to t-acid metarhodopsin in alkaline solution, their patterns of spectral changes and the wavelengths of the isosbestic points were similar to each other at a given temperature. However, the wavelength of the isosbestic point of these transformations varied with the temperature, that is, approx. 490 nm at −1.6°C and approx. 480 nm at 6.8°C. Generally, isosbestic points are found when one substance is transformed to another. If the absorband and wavelength of absorption maximum of these two species do not change with temperature, wavelength of the isosbestic point remarks constant at different temperatures. There are several possible reasons for the shift of the wavelength of the isosbestic point with temperature. Firstly, transient spectral changes at −1.6°C or 6.8°C may represent more than one transformation. The isosbestic point of the spectral change at 6.8°C was not as clear as that for the spectral change at −1.6°C. Accordingly, at an early stage in the spectral changes at 6.8°C there may take place not only the transformation of mesorhodopsin to acid metarhodopsin but also that of lumirhodopsin to mesorhodopsin.

A second possibility is that the transient spectra in the figures may be contaminated with another intermediate whose chromophore was not in an all-*trans* form. Yoshizawa et al. [17] showed that the chromophore of intermediate *P*-465 may be of 7-*cis* retinal form. Therefore, the transient spectra at −1.6°C may be contaminated by a *cis*-form intermediate like *P*-465.

The intermediate process of mesorhodopsin to the final photoproduct of octopus rhodopsin was the same as for squid rhodopsin shown by Ebina et al. [13,14]. T-acid metarhodopsin was converted from mesorhodopsin and some of the converted t-acid metarhodopsin was then transformed to alkaline metarhodopsin reaching a tautomeric equilibrium which determined the pH of the solution.

The intermediate process of mesorhodopsin to the final photoproduct was dependent on the pH of the solution. Acid metarhodopsin was directly converted in acid solution. In alkaline solution, t-acid metarhodopsin was converted from mesorhodopsin and the converted t-acid metarhodopsin was then transformed to alkaline metarhodopsin reaching a tautomeric equilibrium which was determined by the pH of the solution.

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