DYNAMIC ASPECTS OF METARHODOPSIN FORMATION IN PHOTOCHEMICAL CYCLE OF EXTRACTED OCTOPUS RHODOPSIN

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Summary Changes in the difference spectrum of the octopus rhodopsin solution with pH 10.3 were studied at about 2°C by applying the flash photolytic technique. The main peak (about 520 nm) in the difference spectrum at 10 msec after a flash coincided with the peak of the calculated difference spectrum between rhodopsin and acid metarhodopsin. It was suggested that in the octopus rhodopsin cycle the acid metarhodopsin may be a precursor of the alkaline metarhodopsin even in alkaline solution.

The vertebrate rhodopsin bleaches after irradiation of light *via* a series of intermediates (bathorhodopsin, lumirhodopsin, metarhodopsin I, II, and pararhodopsin) to the final products, retinal and opsin (see YOSHIZAWA, 1972). On the other hand, a number of invertebrate rhodopsins are photoconverted in the light to metarhodopsins, which release retinal only very slowly (see GOLDSMITH, 1972). A certain physiologically active intermediate has been reported to play an important role in photoexcitation in the invertebrate visual cell. NOLTE and BROWN (1972) observed in the UV-sensitive visual cell of *Limulus* median ocellus that the photoreceptor membrane is depolarized by UV illumination and the UV-elicited depolarization can be repolarized by a superposed visible light of long wavelength, and that the spectral sensitivity curve of the repolarizing response peaks at 480 nm. From these findings they introduced a hypothesis that the photo-induced reversal change in the membrane potential was the results of the photo-interconversion between the UV-sensitive rhodopsin and a thermally stable intermediate (M 480).

HAMDORF and SCHWEMER (1971) have revealed in their studies on the UV cells of the *Ascalaphus macaronius* that an intermediate which has absorption

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maximum at around 480 nm is an acid form of metarhodopsin. Then it may be considered that the active intermediate (M 480) is also acid form of metarhodopsin and that, at least in some invertebrate visual cells, the essential processes for generation and recovery of the photoreceptor potential may couple with the photo-interconversion between rhodopsin and acid metarhodopsin.

The chemical characteristics of the metarhodopsins in invertebrate rhodopsin cycle have been most extensively studied in the cephalopod. Both of two forms of metarhodopsin, acid and alkaline metarhodopsins, are stable in solution below 20°C (HUBBARD and ST. GEORGE, 1958; KROPF *et al.*, 1959; YOSHIKAWA and WALD, 1964). These intermediates have been believed to be in a thermal tautomeric equilibrium which depends upon the temperature, pH and ionic composition of the solution (MATTHEWS *et al.*, 1963).

In the present experiment the transient changes in optical absorption spectrum of the octopus rhodopsin solution were observed by the technique of flash-photolysis in order to study the process of the formation of acid metarhodopsin at around 2°C. It was found that acid metarhodopsin is photoconverted from rhodopsin independently of pH of the solution. Some of the converted acid metarhodopsins are then transformed to metarhodopsin of alkaline form to reach a tautomeric equilibrium which is decided by pH of the solution.

MATERIALS AND METHODS

Large-eyed octopuses were used for the experiments. The animals (species not yet identified and locally called as *amadako*) were captured in the sea off the coast of Fukushima Prefecture in the northeast district of Japan. The absorption maxima of rhodopsin, acid metarhodopsin and alkaline metarhodopsin are 470, 495, and 370 nm, respectively (Suzuki, T., personal communication).

Rhodopsin was extracted from eyes which were enucleated from dark-adapted animals. Omochrome and salts were removed by passing the crude rhodopsin solution (2% digitonin in phosphate buffer, pH 6.1) through DEAE and Sephadix G-25 columns (ERHARDT *et al.*, 1966; SUZUKI *et al.*, 1972). The solution was freeze dried in the presence of dim red light and stored in a freezer. Before each experiment, the rhodopsin solution was prepared by following ways. The stored sample (50 or 70 mg) was dissolved in the 10 ml of phosphate buffer at pH 6.1. The pH of the solution was then increased to 10.3 by adding some grains of Na_2CO_3 to phosphate buffer. The pH of the solution was recorded for observing the change in pH following photoreactions of rhodopsin, and the change was less than 0.1. The temperature was monitored by a thermister immersed in the solution.

The schematic diagram of the optical arrangement and the recording system is represented in Fig. 1. The rhodopsin solution in a sample cell (C1 ; $2 \times 0.8 \times 2 \text{ cm}^3$) was cooled down to about 2°C by steeping the cell in ice cold water in a trans-

Fig. 1. Schematic diagram of the apparatus. C1: sample cell ($2 \times 0.8 \times 2 \text{ cm}^3$), C2: dewar, C3: water cell, Xe: xenon flash lamp for exciting light, CON: oil condenser bank ($6 \mu\text{F}$), HV: high voltage power supply for Xe, L1, L2: condenser lenses, F1: yellow glass filter (Toshiba VY-46), F2: heat filter, F3: interference filter, M: monochromator (band pass; 5.6 nm), PM: photomultiplier (HTV R-106), W: tungsten lamp for measuring beam (40 W), PS: regulated power supply for W, B1, B2, B3: light baffles, PD: photodiode, MO: monitoring system for intensity of exciting light, CRO: oscilloscope, T: trigger circuit, S3: shutter.

parent dewar (C2) flask. The dewar flask was placed in a cubic glass cell (C3) filled with water to avoid the refraction of the measuring beam from the round surface of the dewar flask.

A light beam from a xenon lamp (Xe; 300 joules, with half duration of about $300 \mu\text{sec}$) was used as an exciting light. The beam was focussed on a sample cell (C1) through a set of condenser lenses (L1, L2), a heat filter (F2) and a yellow glass filter (F1; Toshiba VY-46). For measuring the intensity of the transmitting light through the sample cell, another light beam from a tungsten lamp (W) was set at a right angle to the axis of the exciting beam. An interference filter for a selected wavelength (F3) was placed in front of the sample cell, and a shutter (S3) was opened only during the measurement to minimize the photoreaction by the measuring beam. A Bauch and Lomb grating monochromator (M; band pass, 5.6 nm) was interposed between the sample cell and photomultiplier (PM; HTV-R 106). The wavelength of the measuring light was changed by exchanging the interference filter and by adjusting the wavelength of the monochromator.

Stray light from the exciting flash was minimized by light baffles (B1, B2, B3). The intensity of each flash was monitored by a photodiode (PD) and the fluctua-

tion of light was found to be within 5%. The output signals from the photomultiplier were displayed on an oscilloscope (CRO) and photographically recorded. The xenon flash, the oscilloscope and a monitoring system (MO) were triggered by the same pulse from a trigger circuit (T).

RESULTS AND DISCUSSION

In order to study the transient change in the density spectrum of the rhodopsin solution, the change in intensity of the transmitting light through a sample was recorded after an exciting flash at several wavelengths. Figure 2 shows examples of the transient change in intensity of the transmitting light at 380 nm, 490 nm, and 560 nm through the octopus rhodopsin solution at pH 10.3.

A monotonous decrease in intensity of the measuring light with time after a flash (increase in absorption) was observed at 380 nm (Fig. 2A). This change in alkaline solution may be attributed mainly to the transformation of alkaline metarhodopsin which has an absorption maximum at 370 nm. At wavelengths longer than 450 nm, a monotonous decrease in absorption was preceded by a transient increase in absorption of the measuring light (Fig. 2B and Fig. 2C). The transient increase peaked at about 10 msec after the flash. These results would suggest that the rhodopsin is converted to alkaline metarhodopsin *via* an intermediate with a rather long lifetime and a larger extinction coefficient at around 500 nm than that of rhodopsin.

Fig. 2. Time course of changes in light transmission at 380 nm, 490 nm, and 560 nm through photoexcited rhodopsin solution (pH; 10.3) following an exciting flash. Traces are read from left to right. A downward deflection indicates increased transmission, and upward deflection indicates decreased transmission. Horizontal bars in each picture show initial (I) and final (F) levels of transmission, respectively. Relative intensity of exciting light, A, 1.00; B, 1.01; C, 0.99.

To identify the intermediate in question, the difference spectra were obtained by measuring the change in optical density (ΔOD) before and after the illumination. The ΔOD at each wavelength was calculated by subtracting $\log I(t)$ from $\log I(t=0)$, where $I(t=0)$ and $I(t)$ represent the intensities of the transmitting light before and t sec after the flash, respectively.

Fig. 3. Difference absorption spectra. A: Points marked by ● (0.5 wt. %) and by ○ (0.7 wt. %) indicate change in optical density (ΔOD) during 100 sec after a flash. Vertical bar attached to each point shows the range of experimental error. Solid curve represents difference spectrum (DEC) between rhodopsin and alkaline metarhodopsin. Left ordinate indicates optical density for ΔOD and right ordinate indicates extinction coefficient for DEC. $DEC/\Delta OD$ is $3 \times 10^6 \text{ cm}^2/\text{mol}$. B: Each point indicates ΔOD during 10 msec after a flash. Solid curve is DEC between rhodopsin and acid metarhodopsin. Dashed curve shows DEC between rhodopsin and lumirhodopsin of *Octopus vulgaris*.

Figure 3A shows ΔOD during 100 sec after the flash as a function of the wavelength. Filled and open circles were obtained at the concentration of 0.5 and 0.7 wt. %, respectively. A vertical bar attached to each circle shows the range of the experimental error mainly due to the fluctuations of the signal in the measuring system. These plots indicate an increase in optical density at 380 nm and a decrease at around 480 nm in alkaline solution during 100 sec after the flash. Then the difference spectrum in optical density was compared with the difference spectrum between rhodopsin and alkaline metarhodopsin. The latter spectrum was calculated from the difference in extinction coefficient (will be called hereafter DEC). The computed DEC was superposed by a solid curve in Fig. 3A. The scale for the DEC was drawn in the right ordinate so as to match both spectra at 510 nm. General shapes of the spectrum in ΔOD and that in DEC are in substantial agreement with each other. This agreement may manifest the formation of alkaline metarhodopsin as a stable product in the alkaline solution as reported by static experiments (HUBBARD and ST. GEORGE, 1958).

Filled and open circles in Fig. 3B show the ΔOD 's for 10 msec after the exciting flash. In this figure the plots exhibit a predominant increase in the optical density at around 520 nm. It was suggested from this observation that the flash

illumination develops an intermediate which has a higher extinction coefficient than that of rhodopsin at around 520 nm during 10 msec after the flash.

The DEC between rhodopsin and acid metarhodopsin of *amadako* was superposed in Fig. 3B by a solid curve. As shown in the figure, the plots for ΔOD are distributed on the curve through whole wavelengths. Although it seems clear that the transient increase in absorption at around 520 nm (see Fig. 2B, C) is the result of the acid metarhodopsin formation even in alkaline solution, another possibility should be considered before drawing this conclusion.

If an intermediate which is transformed prior to the metarhodopsins in the photochemical cycle has a similar absorption spectrum to that of acid metarhodopsin, the same result may be obtained by the formation of this intermediate. The possible intermediates that have been reported are bathorhodopsin and lumirhodopsin (YOSHIZAWA and WALD, 1964).

The decay time of the bathorhodopsin of cattle at room temperature is about 50 nsec (ROSENFELD *et al.*, 1972; BUSCH *et al.*, 1972), and the squid bathorhodopsin has been suggested to have a narrower stability range than that of cattle bathorhodopsin (YOSHIZAWA and WALD, 1964). Then it is assumed that the bathorhodopsin formation could not be detected in the present experiments because of far larger time resolution of the measuring system than the decay time.

As for the octopod lumirhodopsin, only the absorption spectrum of this intermediate of *Octopus vulgaris* has been reported (KROPF *et al.*, 1959), and there is no available information about the absorption spectrum of the intermediate of *amadako*. The DEC between rhodopsin and lumirhodopsin of *Octopus vulgaris* was represented by a dashed curve in Fig. 3B for reference. The shape of the dashed curve is different from that of the solid curve especially at the shorter wavelengths as shown in the figure.

The absorption spectrum of lumirhodopsin of *Octopus vulgaris* may be somewhat different from that of *amadako*, and the dashed curve for *Octopus vulgaris* would be inadequate for a precise comparison. However, the precursor of lumirhodopsin (bathorhodopsin) should be unstable at around 2°C as described earlier, and the time course of the lumirhodopsin formation may be very rapid in the octopus rhodopsin cycle comparing with the rise time of the transient increase in absorption which is shown in Fig. 2B (more than 10 msec). Then, the possibility that the transient increase at around 520 nm is due to the formation of lumirhodopsin would be safely excluded. Therefore it seems plausible that the transient increase in absorption at around 520 nm is caused by the formation of acid metarhodopsin even in alkaline solution.

Assuming that the acid metarhodopsin is transformed antecedently to the alkaline metarhodopsin in alkaline solution, one might expect that the slow decrease in absorption following the transient increase is greatly affected by decreasing the pH of the solution as a result of the accumulation of acid metarhodopsin. In fact, as shown in Fig. 4, the time course of the change in absorp-

Fig. 4. Time course of changes in light transmission through photoexcited rhodopsin solution at pH 5.4. Trace is read from left to right. An upward deflection indicates decreased transmission. Horizontal bars show initial (I) and final (F) levels of transmission respectively.

tion of acid solution is different from that of alkaline solution (see Fig. 2B). The trace in Fig. 4 indicates the suppression of the alkaline metarhodopsin formation.

On the basis of these findings, following conclusion may be made. The acid metarhodopsin is photoconverted from rhodopsin independently of the pH of the solution. In alkaline solution, the acid metarhodopsin which is transformed from rhodopsin at early stages of the photochemical reactions is converted to the alkaline metarhodopsin during 100 sec after the flash. In other words, the acid metarhodopsin is a precursor of the alkaline metarhodopsin even in alkaline solution.

Recently Tsukahara *et al.* (manuscript in preparation) have observed, in their studies on the early receptor potential of isolated octopus retina, that the acid metarhodopsin is photoconverted from rhodopsin independently of the pH of the perfusing solution. They speculated that at least in the viable retina the stable photoproduct of rhodopsin may be primarily acid form of metarhodopsin. This view may be consistent with the present conclusion.

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