A NEW INTERMEDIATE BETWEEN LUMIRHODOPSIN AND METARHODOPSIN IN SQUID

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Received 29 May 1975

1. Introduction

Squid rhodopsin, like vertebrate rhodopsin, displays positive circular dichroism (CD) at wavelengths corresponding with its α- and β-absorption bands, due to the binding of 11-*cis* retinal to opsin [1]. It has been reported, however, that squid acid and alkaline metarhodopsins do not display any CD in their main absorption bands [2]. Recently it was found that catttle pararhodopsin displays no CD [3,4] but metarhodopsins I and II do positive CD [3–5]. It is of interest to investigate whether or not an intermediate, perhaps possessing a positive CD as do the vertebrate metarhodopsins, may appear in the photo-conversion of squid rhodopsin.

2. Materials and methods

Fresh squid (*Todarodes pacificus*) eyes were frozen for storage. According to the method described by Hubbard and St. George [6], the rhabdomeres were isolated from the retinas by the sucrose floating method. Then rhodopsin in the rhabdomeres was extracted with 2% digitonin in 0.1 M sodium carbonate buffer (pH 10.1) or 0.1 M phsophate buffer (pH 5.5). The extracts contained no retinochrome, since addition of hydroxylamine (in a final concentration of 0.1 M) to the extracts caused no decrease in absorbance in the visible region except for the effect of dilution. After concentration of the rhodopsin extract by ultracentrifugation at 10 500 *g* for more than 12 hr, glycerol was added to the preparation in a final concentration of 75%. The mixture thus obtained was used as a sample for measuring the absorption and CD spectra at low temperatures.

For these measurements a specially designed glass cryostat with quartz windows was used, as described by Yoshizawa [7]. Absorption spectra were measured in a Hitachi 323 recording spectrophotometer, and CD spectra in a Jasco J-20 recording spectropolarimeter.

3. Results

Fig.1 shows the spectral change in the course of conversion of lumirhodopsin into acid metarhodopsin. When rhodopsin (pH 5.6) (curve 1) was irradiated at 437 nm and −85°C, the spectrum shifted to longer wavelengths, indicating formation of lumirhodopsin. Prolonged irradiation yielded a photo-steady state mixture composed of rhodopsin, isorhodopsin and lumirhodopsin (curve 2). Immediately after warming this mixture to −5°C, it was re-cooled to −85°C, and the spectrum was re-measured. It has shifted to shorter wavelengths and increased in absorbance (curve 3). Further warming to 10°C followed by cooling to −85°C, resulted in a decrease of absorbance and a small shift to longer wavelengths (curve 4). Since the product formed at 10°C is what has been called acid metarhodopsin, curve 3 must involve a prior intermediate, different from acid metarhodopsin in stability, extinction coefficient and absorption maximum.

In order to determine the absorption and CD spectra of the new intermediate, an experiment was performed using an alkaline preparation (pH 10.1), since alkaline metarhodopsin possesses no absorbance and CD at longer wavelengths than 440 nm. Rhodopsin (fig.2, curve 1) was irradiated at longer wavelengths than 530 nm and −40°C for about

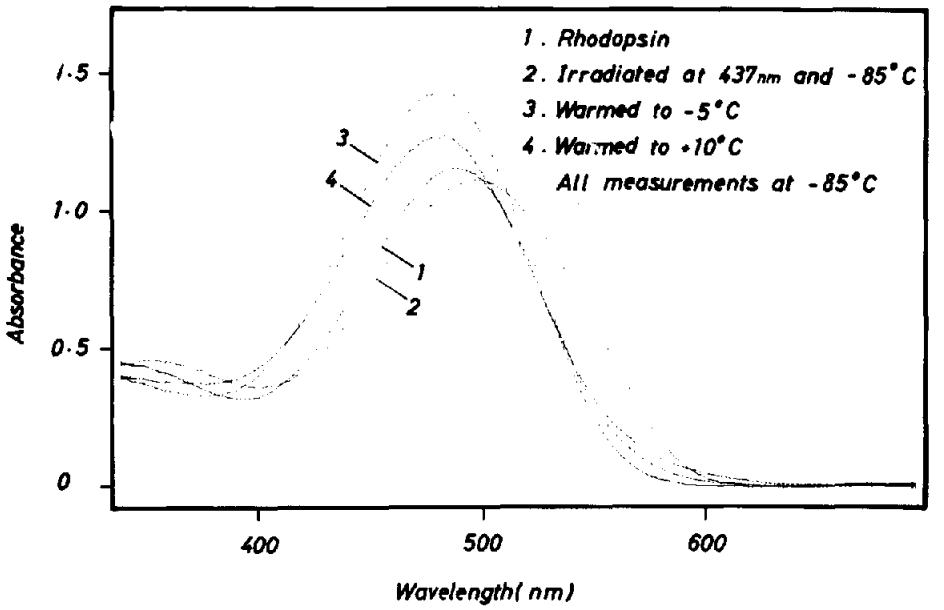

Fig.1. Formation of the new intermediate (intermediate LM) in acid solution (0.1 M phosphate buffer, pH 5.6). The rhodopsin–glycerol mixture (1:3) was cooled to −85°C (curve 1) and irradiated at 437 nm for 171 min (curve 2), forming a photo-steady state mixture composed of rhodopsin, isorhodopsin and lumirhodopsin. Then the mixture was warmed to −5°C, converting the lumirhodopsin to intermediate LM, and then re-cooled to −85°C (curve 3). Finally the preparation was warmed to 10°C to change intermediate LM to acid metarhodopsin and then re-cooled to −85°C (curve 4).

3 hr, resulting in a mixture of rhodopsin, isorhodopsin and the intermediate with a concomitant of lumirhodopsin, the latter of which was detected by CD spectrum. In a preliminary experiment, we confirmed that the irradiation at this temperature prevents any formation of metarhodopsins. Then, the preparation was warmed to −20°C and incubated at this temperature for 36 hr in the dark, in order to complete the conversion of lumirhodopsin into the new intermediate (curve 2). On warming the preparation to 5°C and keeping at this temperature for 48 hr, the peak near 475 nm fell and a new peak appeared in the near ultraviolet region, indicating the conversion of the new intermediate into alkaline metarhodopsin (curve 3).

To obtain the spectrum of the new intermediate, we began to calculate the difference spectrum between the new intermediate and alkaline metarhodopsin by subtraction of curve 3 from curve 2. Then, the percentage conversion of rhodopsin into the new intermediate was estimated from the absorbances at 496 nm (an isosbestic point at −196°C between rhodopsin and isorhodopsin) of curve 1, 3 and 4 in fig.2 [7]. Since one brings some error in determination of wavelength of the isosbestic point at −40°C, we regarded it as that at −196°C, which one can estimate exactly. Thus, the spectrum of the new intermediate can be obtained by addition of the spectrum of alkaline metarhodopsin to the difference spectrum corrected by the percentage of conversion.

Fig.3 shows the absorption and CD spectra of lumirhodopsin, the new intermediate, and acid and alkaline metarhodopsins. The spectrum of lumirhodopsin was calculated by the same method as that of the new intermediate. Lumirhodopsin has its main absorption peak at 506 nm; there is a negative CD peak near 480 nm, a positive CD peak near 360 nm, and then the CD rises to 300 nm. The inconsistency in λ_{max} between the absorption and CD spectra suggests that the α-band of lumirhodopsin is composite, involving more than one electronic transition. The absorption spectrum of the new inter-

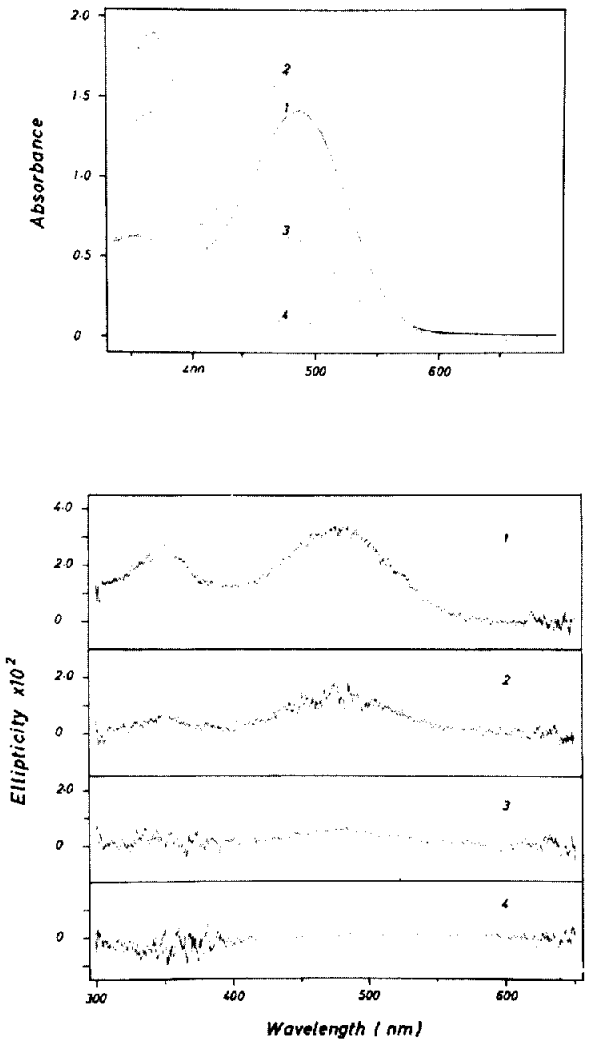

Fig.2. Formation of the new intermediate (intermediate LM) in alkaline solution (0.1 M sodium carbonate buffer, pH 10.1). Absorption and CD spectra are shown in the upper and lower figures respectively. A rhodopsin–glycerol mixture (1:3) was cooled to −40°C (curves 1). Then the preparation was irradiated at wavelengths longer than 530 nm for 2.9 hr in order to convert rhodopsin to a mixture of rhodopsin, isorhodopsin and intermediate LM with some lumirhodopsin. For removing the lumirhodopsin from the preparation, it was incubated at −20°C for 36 hr and then re-cooled to −40°C to measure the spectra (curves 2). For conversion of intermediate LM to alkaline metarhodopsin, the preparation was warmed to 5°C, and then incubated below 5°C for 48 hr. The spectra were measured at −40°C (curves 3). Finally, the preparation was completely bleached with longer wavelengths than 500 nm at 5°C, and then re-cooled to −40°C to measure the spectra (curves 4).

mediate shows λ_{max} at 475 nm and the CD spectrum displays a positive band at the same wavelength as the absorption band. We shall call the new intermediate 'Intermediate LM' tentatively. In such squid preparations, the sign of CD is inverted at the stage at which lumirhodopsin goes over to intermediate LM. We have confirmed that acid and alkaline metarhodopsins have no CD near their main absorption bands (480 and 367 nm at 2°C respectively).

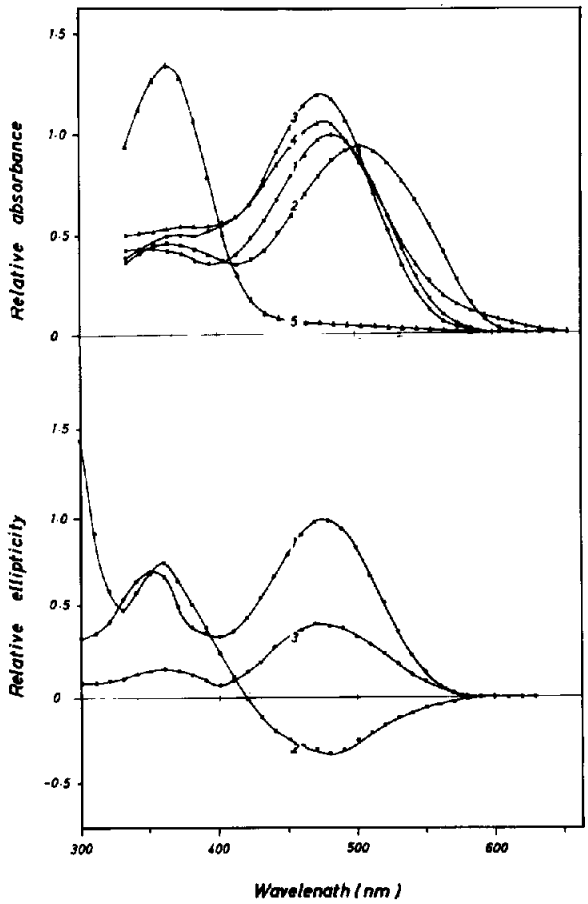

Fig.3. The absorption (above) and CD (below) spectra of rhodopsin and its intermediates of bleaching. The spectra of the intermediates are all drawn relative to the absorbance or the ellipticity of rhodopsin at the temperature at which its spectrum was measured. Curve 1 is the spectra of rhodopsin at −40°C (λ_{max}; 486 nm); curve 2, lumirhodopsin at −85°C (λ_{max}; 506 nm); curve 3, intermediate LM at −40°C (λ_{max}; 475 nm); curve 4, acid metarhodopsin at −40°C (λ_{max}; 482 nm) and curve 5, alkaline metarhodopsin at −40°C (λ_{max}; 368 nm).

The absorption spectrum of acid metarhodopsin (pH 5.5) in 75% glycerol peaks at λ_{max} 480 nm, i.e., at a shorter wavelength than that in phosphate buffer (488 nm). The addition of 75% glycerol to the digitonin extract containing acid metarhodopsin not only caused λ_{max} to shift toward the blue, but also a remarkable decrease of extinction and broadening of the shape of the spectrum. Such effects of glycerol have never been observed on rhodopsin.

4. Discussion

At the annual meetings of the Biophysical and Zoological Societies of Japan held at Sapporo from September 29 to October 4 in 1974, we and two other groups gave papers on the new intermediate. One group (Y. Ebina, Y. Tsukahara and N. Nagasawa) reported that their new intermediate (λ_{max} 475 nm), which they call D_{460}, was detected by means of flash photolysis in the process of conversion of lumirhodopsin into acid metarhodopsin; and that acid metarhodopsin is also formed transiently from the new intermediate in alkaline solution. We could not detect acid metarhodopsin under alkaline conditions, probably owing to its rapid transformation to alkaline metarhodopsin, which one can only observe within 200 s after a flash at 5°C according to their report. The absorption maximum of intermediate D_{460} is compatible with intermediate LM in wavelength and extinction coefficient. Though they showed that D_{460} has a lower extinction coefficient than acid metarhodopsin compared with our results, the discrepancy is due to the decrease of extinction of acid metarhodopsin caused by glycerol, since their and our preparations contained 25% and 75% glycerol respectively. Another inconsistency is seen in the shape of spectrum of D_{460}, which has a shoulder on the long-wavelength side of peak, probably owing to the presence of some lumirhodopsin. The other group of workers (T. Suzuki, K. Azuma and M. Azuma) reported the absorption and CD spectra of a new intermediate. They call it intermediate A (λ_{max} approx. 485 nm), which scarcely shows any CD in the visible region, whereas intermediate LM displays a positive CD in the visible region. This inconsistency in CD as well as in λ_{max} may also be caused by some contamination by lumirhodopsin in their sample. They prepared the sample containing the intermediate A by irradiating squid rhodopsin in 66% glycerol at dry ice–acetone temperature, and warming to −30°C. We find that more than 2 hr incubation at −30°C is needed to completely eliminate lumirhodopsin in preparations produced by irradiating squid rhodopsin in 66% glycerol mixtures at temperatures below −35°C. The sample which we used to calculate the spectrum was incubated long enough to remove lumirhodopsin completely.

It has been assumed that vertebrate metarhodopsin I or II may play an important role in generating receptor potentials in visual cells [8]. Since intermediate LM is somewhat analogous to vertebrate metarhodopsin I in the bleaching sequence of squid rhodopsin, it may have a role in the squid photoreceptive mechanism.

Acknowledgements

These investigations were partly supported by the grants from the Japanese Ministry of Education and Toray Science Foundation to T. Yoshizawa, and from the Sakkokai Foundation to F. Tokunaga. We should like to express our thanks to Professor G. Wald for reading the manuscript.

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