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STUDIES ON CEPHALOPOD RHODOPSIN

CONFORMATIONAL CHANGES IN CHROMOPHORE AND PROTEIN DURING THE PHOTOREGENERATION PROCESS

TATSUO SUZUKI[a,*], MICHIO SUGAHARA[a], KATSU AZUMA[b], MASAMI AZUMA[c], YOSHIRO SAIMI[a] and YUJI KITO[a]

[a]*Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka*; [b]*Department of Biology, Osaka Medical College, Takatsuki, Osaka, and* [c]*Department of Health Science, Osaka Kyoiku University, Tennoji, Osaka (Japan)*

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SUMMARY

The ultraviolet absorbance of squid and octopus rhodopsin changes reversibly at 234 nm and near 280 nm in the interconversion of rhodopsin and metarhodopsin. The absorbance change near 280 nm is ascribed to both protein and chromophore parts. Rhodopsin is photoregenerated from metarhodopsin via an intermediate, P380, on irradiation with yellow light ($\lambda > 520$ nm). The ultraviolet absorbance decreases in the change from rhodopsin to metarhodopsin and recovers in two steps; mostly in the process from metarhodopsin to P380 and to a lesser extent in the process from P380 to rhodopsin. P380 has a circular dichroism (CD) band at 380 nm and its magnitude is the same order as that of rhodopsin. Thus it is considered that the molecular structure of P380 is close to that of rhodopsin and that the chromophore is fixed to opsin as in rhodopsin. In the change from metarhodopsin to P380, the chromophore is isomerized from the all-*trans* to the 11-*cis* form, and the conformation of opsin changes to fit 11-*cis* retinal. In the change from P380 to rhodopsin, a small change in the conformation of the protein part and the protonation of the Schiff base, the primary retinal–opsin link, occur.

INTRODUCTION

Cephalopod rhodopsin has 11-*cis* retinal as its chromophore, and light isomerizes it into the all-*trans* configuration. The photochemical reaction is followed by thermal ones including conformational changes in the protein part, opsin. The metarhodopsin of cephalopod is stable below 15 °C and can be reversed to rhodopsin by light [1]. In the photoregeneration of squid rhodopsin, we found an intermediate (P380) which is produced from metarhodopsin by irradiation with yellow light and

* Present address: Department of Pharmacology, Hyogo Medical College, Nishinomiya, Hyogo (Japan).

has 11-*cis* retinal as chromophore [2, 3]. The production of P380 is remarkable in the presence of borate ions, but in the absence of borate ions only small amounts of P380 are produced [3]. P380 reverts to rhodopsin in the dark and its rate constant depends on temperature, pH and solvent composition. We thought in the previous paper [3] that the conformation of opsin changes to fit the 11-*cis* retinal during the change from P380 to rhodopsin. It appears that important information on the structure and function of rhodopsin can be obtained by further studies on the photoregeneration process that passes through P380.

In cattle and squid rhodopsin, reversible ultraviolet absorbance changes have been reported, indicating reversible conformational changes of the protein part in the bleaching and regeneration processes [4, 5]. The first purpose of the present paper is to elucidate the conformation of opsin in P380 by studying the ultraviolet absorbance changes in the photoregeneration process.

The absorption bands of squid rhodopsin in the visible spectrum are optically active and the activity is lost at the metarhodopsin stage [6]. Cattle metarhodopsin and squid retinochrome have all-*trans* retinal as chromophore, and their absorption bands in the visible spectrum are optically active [7, 8]. It is assumed that in these optically active pigments, the chromophore, irrespective of configuration, is fixed to the protein resulting in restriction of its free rotation and is not fixed in such optically inactive pigment as squid metarhodopsin. At what stage is the chromophore fixed to opsin in the photoregeneration process of squid rhodopsin? The second purpose of the present paper is to answer the question whether or not the chromophore of P380 is fixed to opsin as in rhodopsin.

MATERIALS AND METHODS

Octopus variabilis and *Todarodes pacificus* were used as experimental materials The pK of the equilibrium of acid metarhodopsin $\rightleftharpoons$ alkaline metarhodopsin is 7.4 in the octopus and 9.1 in the squid. Extraction and purification of rhodopsin were carried out by the method described in the previous paper [3, 8]. The outer segments of the photoreceptor cells were isolated by floatations with 40 % sucrose, washed, extracted with 2 % digition and purified on a DEAE-cellulose column. Absorption spectra were determined with the Hitachi EPS-2U and the Hitachi 124 spectrophotometers. The temperature of the sample was kept constant by circulating water of the desired temperature through the cell holder. The sample was irradiated by light from a 500-W tungsten lamp which had passed through a glass filter or interference filter. The light was focused by an optical system and led to the sample cell through an optical glass fibre. The measurement of CD was carried out with the JASCO J-20 spectropolarimeter by using a special apparatus for low temperature CD (light path, 15 mm).

Other experimental details are described with the experimental results.

RESULTS

(*1*) *Reversible ultraviolet absorbance changes in the interconversion of rhodopsin and metarhodopsin*

Ultraviolet absorbance changes were studied by using octopus rhodopsin, a

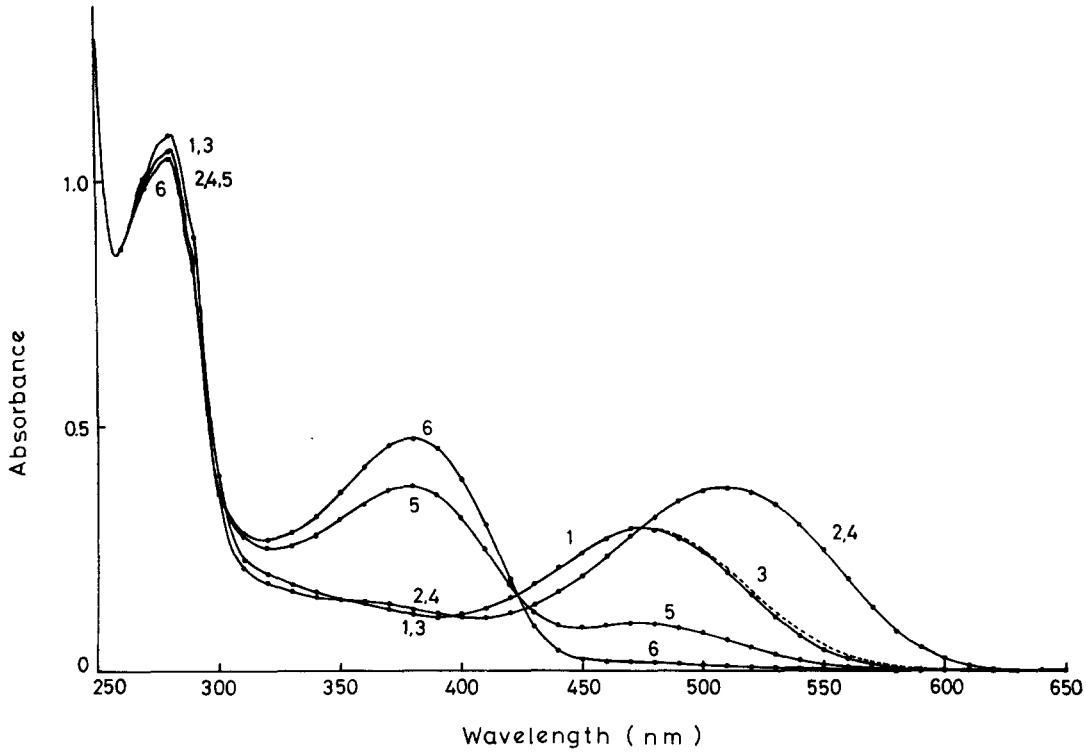


Fig. 1. Absorbance spectra of octopus rhodopsin. Rhodopsin at pH 5.5 (Curve 1) adjusted to this pH by KH_2PO_4 was irradiated with blue light ($\lambda = 440$ nm) for 5 min (Curve 2) and then with red light ($\lambda > 600$ nm) for 5 min (Curve 3). The solution was then re-irradiated with blue light for 5 min (Curve 4) and then the pH was raised to 10.5 by adding grains of Na_2CO_3 (Curve 5). Finally, the solution was irradiated with yellow light ($\lambda > 520$ nm) for 2 min and all rhodopsin converted to alkaline metarhodopsin (Curve 6). Absorbance spectra were determined at 6 °C.

good material for this experiment because the absorption maximum of acid metarhodopsin is located at a much longer wavelength. (Absorption maxima of rhodopsin and acid metarhodopsin are 477 nm and 510 nm, respectively, in *Octopus variabilis*.) Consequently the interconversion of rhodopsin and metarhodopsin can be easily induced by alternate irradiations with blue and red light.

Fig. 1 shows the spectral changes in the interconversion of rhodopsin and acid metarhodopsin. When octopus rhodopsin (Curve 1) is irradiated with blue light ($\lambda = 440$ nm) for 5 min at pH 5.5, a steady-state mixture of rhodopsin and acid metarhodopsin (Curve 2) is formed. The acid metarhodopsin in the mixture reverts to rhodopsin on irradiation with red light ($\lambda > 600$ nm) for 5 min (Curve 3). At the Curve-3 stage the solution contains acid metarhodopsin to an extent of less than 5 % of total pigment. When the solution was then re-irradiated with blue light for 5 min, the absorption spectrum moved to Curve 4, which is identical with Curve 2. Then the solution was brought to pH 10.5, by the addition of small grains of Na_2CO_3, in order to convert acid metarhodopsin to alkaline metarhodopsin (Curve 5). All the rhodopsin remaining was then converted to alkaline metarhodopsin by further

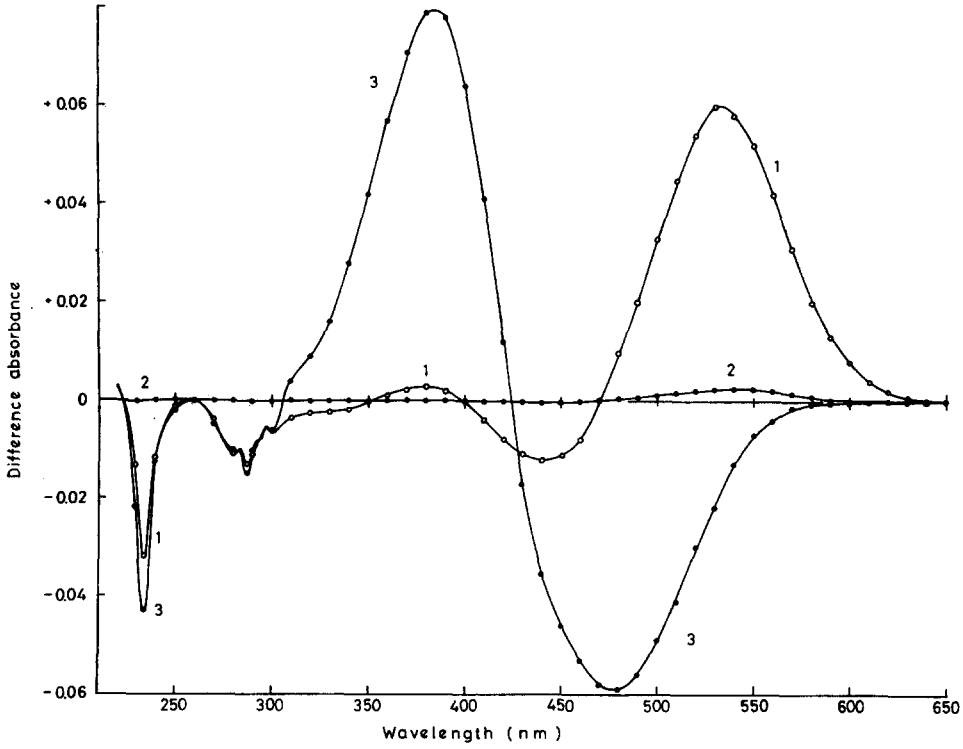


Fig. 2. Difference spectra of octopus rhodopsin. The rhodopsin solution was irradiated with blue light (Curve 1) and then with red light for 5 min (Curve 2) at pH 5.5 adjusted to this pH by KH_2PO_4. Another rhodopsin solution adjusted to pH 10.5 by Na_2CO_3 was irradiated with yellow light for 2 min (Curve 3). These spectra were determined at 6 °C with reference to unirradiated rhodopsin solution.

irradiation with yellow light ($\lambda > 520$ nm), as shown by Curve 6. These results indicate that about 70 % of the initial rhodopsin is converted to acid metarhodopsin in a steady-state mixture (Curves 2 and 4) under the blue light, and almost all the acid metarhodopsin reverts to rhodopsin under the red light (Curve 3). In this interconversion reversible changes of absorbance in the ultraviolet region can be observed.

Fig. 2 shows the difference spectra of octopus rhodopsin on irradiation, as determined with reference to unirradiated rhodopsin. Curve 1 was obtained after irradiation with blue light for 5 min at pH 5.5, and under this condition about 70 % of the rhodopsin was converted to acid metarhodopsin as mentioned above. The small increase in absorbance near 380 nm is ascribable to the production of a small amount of alkaline metarhodopsin. There are two negative bands at 234 nm and around 280 nm in the difference spectrum. The 280-nm band shows three negative peaks, which may be due to aromatic amino acid residues of opsin. It should be remembered that the third band of 11-*cis* retinal (subsidiary band) also contributes to the 280 nm-band (see Discussion). The decrease of absorbance in the region 350–310 nm may be ascribed to the second band (*cis* peak) of the 11-*cis* chromophore. Then after the solution was further irradiated with red light ($\lambda > 600$ nm) for 5 min, Curve 1 changed to Curve 2, indicating that the acid metarhodopsin was almost completely

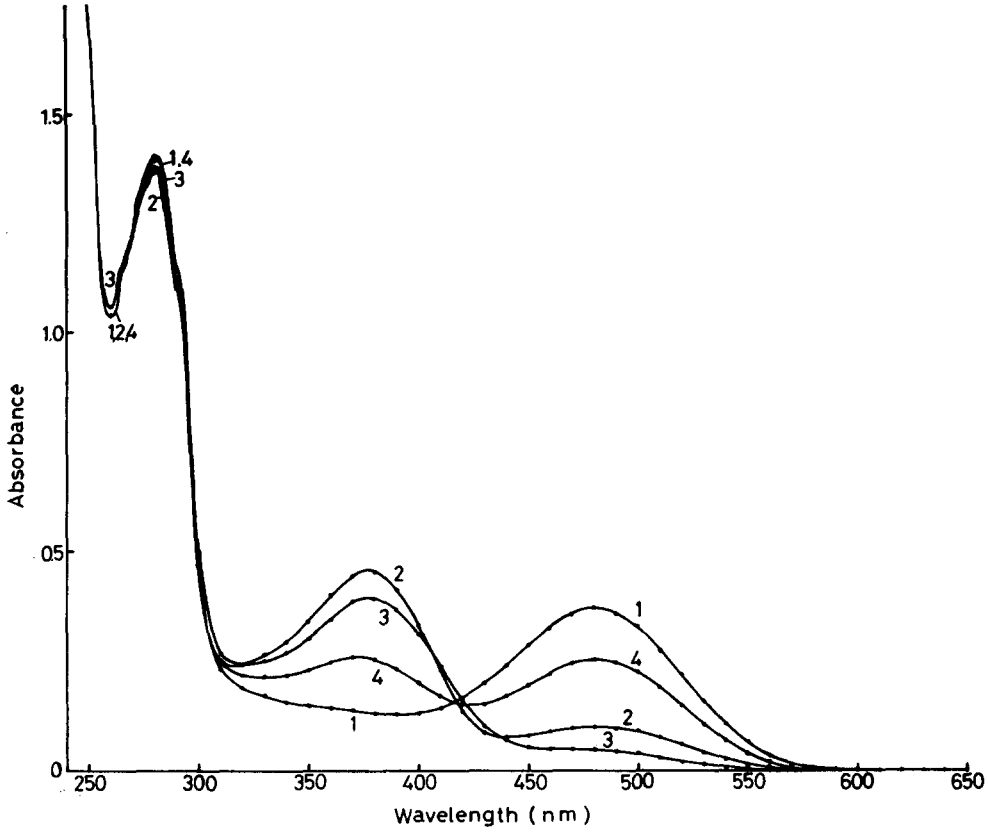


Fig. 3. Absorbance spectra of squid rhodopsin. The rhodopsin solution in 1.0 % digitonin and 0.1 M borate–NaOH buffer at pH 10.2 (Curve 1) was irradiated with yellow light for 10 s (Curve 2) and then for a further 5 min with the same light (Curve 3). The solution (Curve 3) was allowed to stand for 1 h in the dark at 6 °C and then Curve 4 was determined.

converted to rhodopsin. In this reaction, the ultraviolet absorbance recovered completely.

Curve 3 in Fig. 2 is the difference spectrum of octopus rhodopsin when irradiated with yellow light ($\lambda > 520$ nm) for 2 min at pH 10.5, in order to convert all rhodopsin to alkaline metarhodopsin. Absorbance decrease in the ultraviolet region is essentially the same as in the acid condition (Curve 1). The extent of the absorbance decrease at 234 nm in Curve 1 is about 70 % of that of Curve 3, which is in parallel with the amount of metarhodopsin produced by the irradiation. From the results in Fig. 2, it can be said that the changes in ultraviolet absorbance are completely reversible in the interconversion of rhodopsin and metarhodopsin and that the extent of the absorbance change is independent of the pH of the solution.

(2) Ultraviolet absorbance changes in the photoregeneration process passing through P380

The ultraviolet absorbance changes in the two processes of photoregeneration:

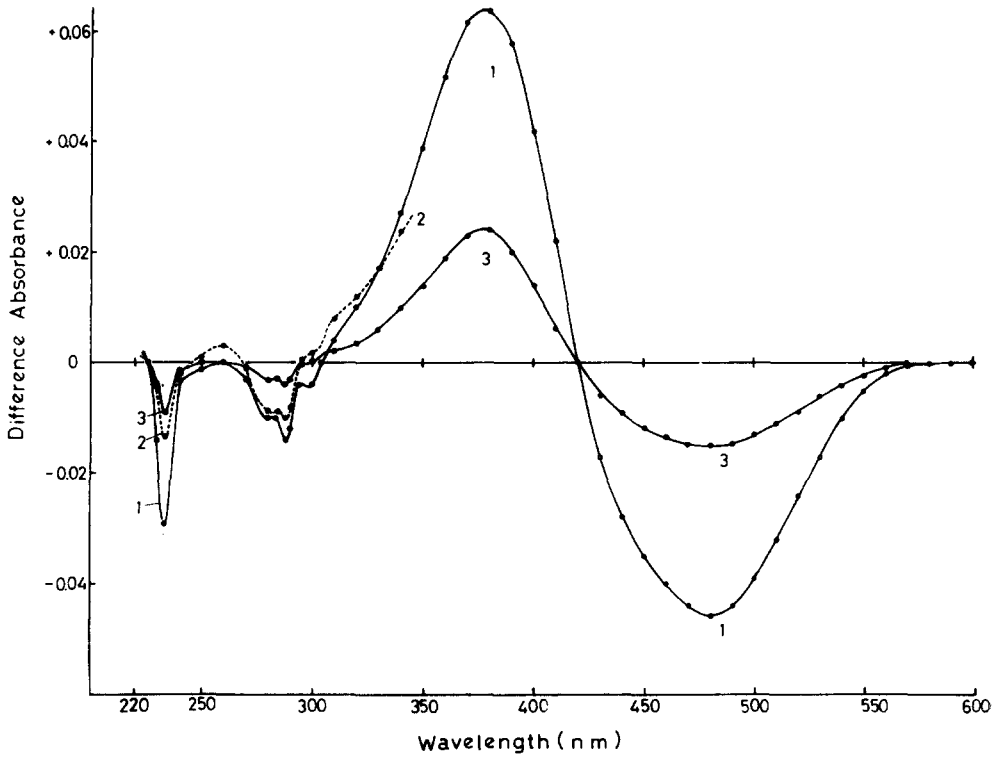


Fig. 4. Difference spectra of squid rhodopsin. The rhodopsin was irradiated with yellow light for 10 s (Curve 1) and then for a further 5 min (Curve 2) in 0.1 M borate–NaOH buffer (pH 10.3) and 0.1 % digitonin at 6 °C. After 1 h incubation in the dark at 6 °C, Curve 3 was determined. These spectra were determined with reference to unirradiated rhodopsin solution.

metarhodopsin → P380 and P380 → rhodopsin, were studied with squid rhodopsin. Fig. 3 shows absorption spectra of squid rhodopsin in the photoregeneration processes. When squid rhodopsin adjusted by the borate–NaOH buffer to pH 10.2 is irradiated for 10 s with yellow light ($\lambda > 520$ nm), 70 % of the rhodopsin is converted to alkaline metarhodopsin as shown by Curve 2. Further irradiation for 5 min results in further bleaching of the rhodopsin and a small decrease in absorbance at 380 nm (Curve 3), showing the production of P380 of which the ε_{max} is lower than that of alkaline metarhodopsin [3]. The P380 reverts to rhodopsin in a subsequent dark reaction. The final solution is a mixture of regenerated rhodopsin and alkaline metarhodopsin (Curve 4). Since the amount of P380 produced by the 5-min irradiation with yellow light is equal to that of the regenerated rhodopsin, its amount is estimated as 70 % of the initial rhodopsin. Reversible changes in ultraviolet absorbance can also be observed in the photoregeneration processes.

We studied the ultraviolet absorbance changes in these processes in detail by the measurement of difference spectra with reference to unirradiated rhodopsin, and the results are shown in Fig. 4. On irradiation of the rhodopsin solution for 10 s with yellow light, 70 % is converted to alkaline metarhodopsin, and the absorbances

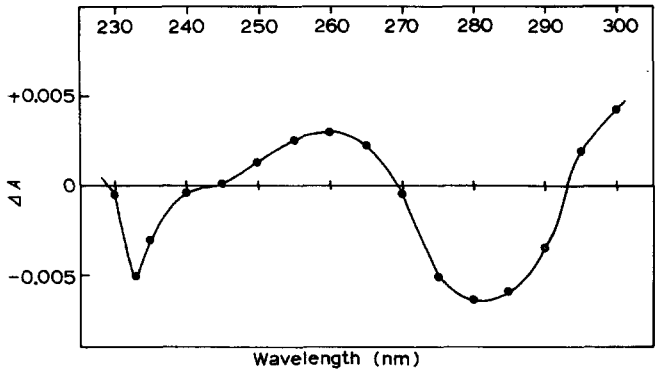


Fig. 5. Difference spectrum between rhodopsin and P380. The spectrum was determined by irradiation of squid rhodopsin solution with yellow light for 5 min. The reference material was a mixture of the regenerated rhodopsin and alkaline metarhodopsin, obtained by 1 h incubation in the dark after 5 min irradiation with yellow light. Experimental conditions as in Fig. 4.

at 234 nm and at around 280 nm decrease (Curve 1 in Fig. 4). Further irradiation of the sample solution with yellow light for 5 min produces P380, the amount of which is 70 % of the initial rhodopsin, and the absorbance at 234 nm largely recovers (85 %). The absorbance at around 280 nm recovers to a small extent and a new positive band at 260 nm appears concomitantly (Curve 2). In the following dark period, the 260-nm band disappears and the absorbance at 280 nm recovers markedly. The extent of absorbance recovery at 234 nm is small (15 %), however. The final solution relates to Curve 3, which is ascribed to the mixture of 30 % alkaline metarhodopsin and 70 % rhodopsin.

The net absorbance changes in the change from P380 to rhodopsin can be obtained arithmetically by subtracting Curve 3 from Curve 2 in Fig. 4. But we obtained it by an experimental procedure as follows. After the experiment yielding Curve 3 in Fig. 4, the reference rhodopsin solution and the sample solution were interchanged. Then the rhodopsin solution was irradiated with yellow light for 5 min and the difference spectrum determined. The contribution of alkaline metarhodopsin to the difference spectrum was thus cancelled out and the net absorbance change could be obtained. Fig. 5 shows the result, indicating the ultraviolet absorbance changes in the change from rhodopsin to P380. This spectrum disappears on incubation in the dark and reappears on re-irradiation. It should be noted that the 260-nm and 280-nm bands are smooth and broad. It seems reasonable to suppose that these two bands are due to the spectral shift of the third band of 11-*cis* chromophore. Next, the time courses of the absorbance changes at 280 nm and 480 nm were recorded, and the results are shown in Fig. 6. These are first-order reactions having the same half-times. The absorbance increase at 280 nm is 15 % of that at 480 nm.

(3) CD changes in the photoregeneration process

The CD spectrum of squid rhodopsin was determined in a solution containing 0.1 M borate–NaOH buffer (pH 10.5) at 2 °C. Squid rhodopsin exhibits two CD bands corresponding to the absorption bands at 480 nm (α-band) and at 340 nm

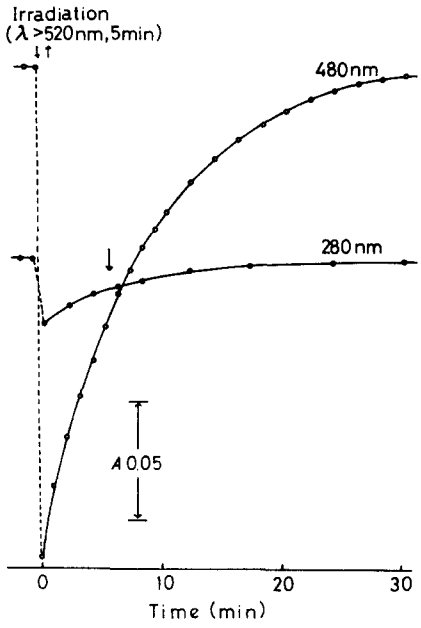


Fig. 6. Time courses of absorbance changes at 280 nm and 480 nm. Squid rhodopsin solution in 0.1 % digitonin and 0.1 M borate–NaOH buffer (pH 10.3) was irradiated with yellow light for 5 min, and then absorbances at 280 and 480 nm were recorded in the dark at 6 °C.

(β-band). The rhodopsin solution was then irradiated with yellow light ($\lambda > 520$ nm) for 5 min, and the CD absorbances around 380 nm and 480 nm were recorded successively. Immediately after the irradiation, the CD absorbance at 480 nm decreased markedly and a new CD absorbance at 380 nm appeared. Alkaline metarhodopsin has absorption maximum at 380 nm like P380. However, the CD absorbance at 380 nm increased by the irradiation is ascribed to P380 because alkaline metarhodopsin is optically inactive [6]. The CD absorbance at 380 nm decreases and that at 480 nm increases with time after irradiation in the dark. The time courses of the CD absorbance changes in the dark are shown in Fig. 7A. When the logarithm of CD absorbance at 480 nm is plotted against time after irradiation, the points lie on a straight line, indicating a first-order reaction with $k = 4 \cdot 10^{-4}\,s^{-1}$. This value is identical to that determined from the absorbance changes at 480 nm under the same experimental conditions. The CD absorbance at 380 nm is derived not only from the CD absorbance of P380 but also from the CD absorbance of the β-band of rhodopsin regenerated in the dark. The extent of the contribution of the regenerated rhodopsin to the CD absorbance at 380 nm is 37 % of the CD absorbance at 480 nm, which is estimated from the CD spectral curves of rhodopsin determined before irradiation and after dark incubation. Subtracting the contribution of the regenerated rhodopsin from the observed CD absorbance at 380 nm, the net CD absorbance of P380 can be obtained and is shown by the dotted curve in the figure. The logarithm of the calculated CD absorbance of P380, also, lies on straight line. We find the CD absorbance of P380 as $4.65 \cdot 10^{-4}$ by extrapolating the straight line to zero time. Since

the amount of P380 produced by irradiation is equal to that of the regenerated rhodopsin, we can estimate the magnitude of the CD absorbance of P380 at 380 nm to be 0.9 times that of rhodopsin at 480 nm. The absorbance spectra of the initial rhodopsin and of the regenerated pigment after this experiment are shown in Fig. 7B. This indicates that 80 % of the initial rhodopsin is regenerated via P380 in this experiment, a value identical with the extent of recovery of the CD absorbance at 480 nm.

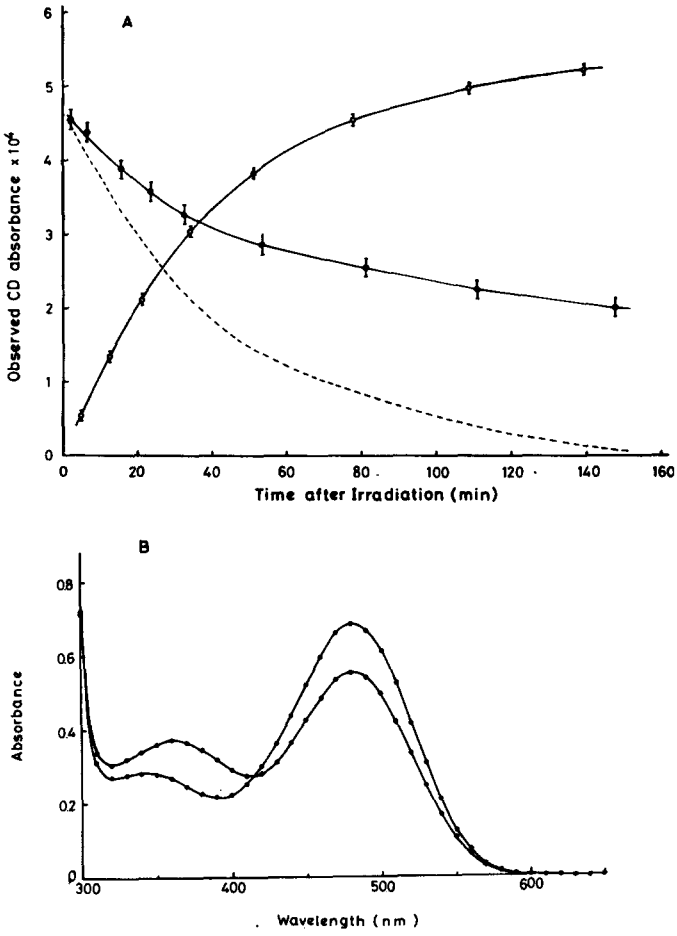
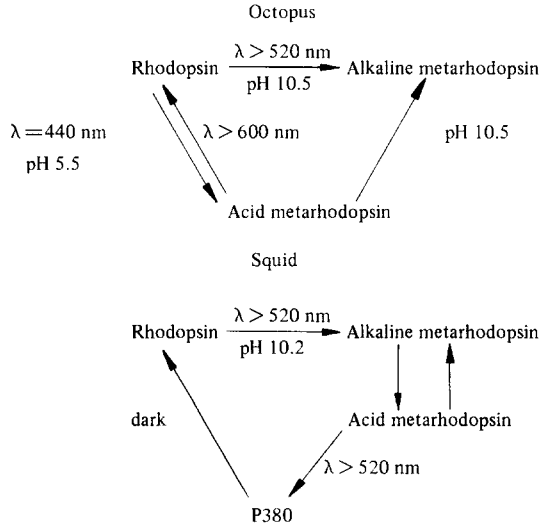


Fig. 7. Time courses of CD absorbances ($\Delta A_{1,r}$) at 380 nm and 480 nm after the irradiation. (A) Squid rhodopsin solution in 0.1 % digitonin and 0.1 M borate–NaOH buffer (pH 10.5) was irradiated with yellow light for 5 min, and then the CD absorbances at 380 nm (●–●) and 480 nm (○–○) were recorded in the dark at 2 °C with a light path of 15 mm. The net CD absorbance of P380 was calculated and is shown (- - -). The CD absorbance at 480 nm was $6.4 \cdot 10^{-4}$ before irradiation and $5.2 \cdot 10^{-4}$ after incubation in the dark. Noise levels are indicated as vertical bars. (B) Absorbance spectra of squid rhodopsin used for the CD measurement. ●–●, before irradiation; ○–○, after irradiation and after 2.5 h incubation in the dark. These spectra were determined at 6 °C with a light path of 10 mm.

DISCUSSION

The reactions of octopus and squid rhodopsin in the present experiment can be summarized as follows:



As reported in the previous paper, P380 is produced from acid metarhodopsin by irradiation with yellow light ($\lambda > 520$ nm) and converts to rhodopsin in the dark [3]. The amounts of P380 accumulated during irradiation is dependent on pH and on the presence of borate ions [3]. The role of borate ions is not yet clear. The pH influences the velocity of conversion of P380 to rhodopsin and the amounts of acid metarhodopsin which absorbs the yellow light. Accordingly, P380 cannot be produced by yellow light at pH values too far from the pK value of the equilibrium of acid metarhodopsin $\rightleftharpoons$ alkaline metarhodopsin. And under neutral and acid condition, the velocity of conversion of P380 to rhodopsin is too fast to be observed. The value of pK is 7.4 in octopus (*Octopus variabilis*) and 9.1 in squid (*Todarodes pacificus*). The reason why P380 is not produced in octopus at pH 10.5 is that there is no acid metarhodopsin. In the presence of borate ions, however, the photoregeneration process via P380 is also clearly observed in octopus in the pH region of 8–9 (unpublished). Accordingly the photoregeneration process of octopus rhodopsin is essentially the same as that of squid rhodopsin.

The absorbance spectrum of rhodopsin is composed of α-, β-, and γ-bands [11]. The α- and β-band are ascribed to the chromophore, 11-*cis* retinal. The γ-band has been considered to be due to the aromatic amino residues in opsin. Recently the contribution of a third band of the 11-*cis* chromophore (subsidiary band) to the γ-band has been suggested by studies on the absorption spectrum of the Schiff base compound [12] and on the difference spectrum of cattle rhodopsin irradiated at a solid CO_2 temperature [13]. The difference spectrum in Fig. 2 shows three negative peaks around 280 nm, where the γ-band is located. The fine structure of the difference spectrum indicates that the absorbance change in this region is partly due to the spectral displacement of aromatic amino residues [4]. The ultraviolet absorbance

change of rhodopsin near 280 nm may be caused partly by the blue shift of aromatic amino residues which are probably exposed to a less polarizable environment in the bleaching process of rhodopsin. However, it is considered that the third band of the 11-*cis* chromophore also contributes to the 280-nm band in the difference spectrum. The absorbance change at 234 nm is not parallel to that at 280 nm as is shown in Fig. 4. The absorbance at 234 nm increases largely in the change from metarhodopsin to P380 and so does the absorbance at 280 nm in the change from P380 to rhodopsin. In the difference spectrum of Fig. 5 the fine structure in the 280-nm band is scarcely observed, suggesting that the absorbance change at 280 nm in this process is due to a broad absorption band. The increase of absorbance at 280 nm in the change from P380 to rhodopsin is accompanied by a decrease of absorbance at 260 nm, and its time course is identical with that of the absorbance at 480 nm (Fig. 6). These facts can be reasonably explained if the third band of the 11-*cis* chromophore is located at 260 nm in P380 and at 280 nm in rhodopsin. The spectral blue shift of the third band of 11-*cis* retinal must be responsible for the absorbance change in the 245–295-nm region during the change from rhodopsin to P380, as shown in Fig. 5.

The results in Fig. 2 indicate that the conformational change of the protein part in the interconversion of rhodopsin and metarhodopsin is completely reversible. Metarhodopsin is more labile than rhodopsin as regards resistance to heat and such reagents as NH_2OH, PCMB [1] and $NaBH_4$ (unpublished). The absorbance decrease in the ultraviolet region and the loss of stability suggests the unfolding of opsin in the change from rhodopsin to metarhodopsin. Thus we can think of the recovery of the ultraviolet absorbance as indicating a refolding of opsin in the photoregeneration process. It was shown by the results in Fig. 4 that 85 % of the absorbance decrease at 234 nm recovers in the change from metarhodopsin to P380, and the remaining 15 % in the change from P380 to rhodopsin. The origin of the absorbance change at 234 nm is more complex than that near 280 nm, because other factors are involved. However, the absorbance change at 234 nm is solely assigned to the conformational change of the protein part and not to the chromophore. Accordingly, we can say that the major conformational change of opsin takes place in the change from metarhodopsin to P380 and only a minor one in the change from P380 to rhodopsin. If this inference is true, it would be expected that P380 would not be produced from metarhodopsin on irradiation at liquid nitrogen temperature, where little conformational change of opsin can occur [9, 10]. In a preliminary experiment, we confirmed no P380 formation at liquid nitrogen temperature on irradiation with light of any wavelength.

The chromophore, retinal, is photoisomerized from all-*trans* to 11-*cis* form in the change from metarhodopsin to P380 [3]. Since the CD absorbance of the chromophore is induced in this process, it may be that 11-*cis* retinal is fixed to an asymmetric part of opsin in P380. In other words, the secondary link that induces the CD absorbance has been formed in P380, and the link is as strong as in rhodopsin because the magnitude of the CD absorbance of P380 is of the same order as that of rhodopsin.

Judging from the ultraviolet absorbance and the CD absorbance, the molecular structure of P380 is close to rhodopsin. Rhodopsin differs from P380 in two points only; one is in the different absorbance maximum and the other is the slight

difference in the conformation of the protein part. The absorbance maximum of rhodopsin is 100 nm longer than that of P380. This red shift is considered to be due to the protonation of a Schiff base, the primary link between retinal and opsin. We cannot exactly know, at present, how different the structure of opsin is between rhodopsin and P380. However, it may be that the conformational change of opsin in the change from P380 to rhodopsin is intimately related to the protonation of the Schiff base link. We suggest that the proton donor exists in the vicinity of the Schiff base link in opsin and that this donor comes into position to donate a proton to the Schiff base when the conformation of opsin changes to fit 11-*cis* retinal [3, 14]. The Schiff base link in rhodopsin has been thought to be embedded in opsin and protected from the attack of such reagents as NH_2OH and $NaBH_4$ [1, 10]. Since the molecular structure of P380 is closer to that of rhodopsin than of metarhodopsin, the Schiff base link in P380 may be embedded inside the opsin as in rhodopsin. This concept is supported by the fact that photoregeneration via P380 is not affected by NH_2OH [2] and $NaBH_4$ (unpublished). In the change from metarhodopsin to P380, the opsin changes its conformation to fit the 11-*cis* chromophore. In the molecule of P380, retinal is primarily bound to opsin through a Schiff base link in unprotonated form, and secondarily through non-covalent interaction which induces the asymmetry in absorption of retinal. In the final change from P380 to rhodopsin, we think the conformation of opsin changes to a more compact structure and a proton is transferred from the donor to Schiff base as mentioned above.

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