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AN INTERMEDIATE IN THE PHOTOREGENERATION OF SQUID RHODOPSIN

TATSUO SUZUKI, MICHIO SUGAHARA AND YUJI KITO

Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560 (Japan)

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SUMMARY

Squid rhodopsin made alkaline with borate–NaOH buffer is bleached by irradiation with yellow light. After irradiation, rhodopsin is regenerated from a substance with an absorption maximum of 380 nm (P_{380}). This regeneration of rhodopsin is scarcely observed in a solution made alkaline with sodium carbonate instead of borate buffer. The reaction velocity of the conversion of P_{380} to rhodopsin is dependent not only on temperature and pH but also on the concentrations of borate buffer and digitonin. P_{380} is produced from acid metarhodopsin during irradiation with yellow light and its chromophore must be 11-*cis*-retinal. This substance with λ_{max} of 380 nm is an intermediate in the photoregeneration process of squid rhodopsin.

INTRODUCTION

In a previous paper[1], we reported the regeneration of squid rhodopsin *in vitro*. When a digitonin extract of squid rhodopsin was made alkaline with borate–NaOH buffer (pH 10.0) and irradiated with yellow light at 5 °C, rhodopsin was bleached and a substance with a λ_{max} of 380 nm was formed. After the irradiation, rhodopsin was regenerated from this substance in the dark. This regeneration process could be observed repeatedly and the amounts of regenerated rhodopsin were regulated by the duration of the irradiation and the temperature. However, when a rhodopsin solution was irradiated for a short time (20 s) and a considerable amount of rhodopsin was bleached, regeneration could not be observed. These observations led us to propose that a substance with λ_{max} of 380 nm is a precursor of rhodopsin. This precursor is produced from metarhodopsin during irradiation with yellow light and is then converted to rhodopsin in the dark. This 380-nm product appears to be distinct from alkaline metarhodopsin which also absorbs maximally at about 380 nm.

When squid rhodopsin is bleached, the final product is acid or alkaline metarhodopsin, the percentage of each depending on the pH of the rhodopsin solution[2]. These two metarhodopsins are in tautomeric equilibrium and can be interconverted by changing the pH of the solution. Hubbard and St. George[2] showed that squid rhodopsin could be photoregenerated from metarhodopsin. According to them, the interconversion between squid rhodopsin and metarhodopsin occurs only during irradiation and no dark reaction is involved. They also reported that the continuous

irradiation of squid rhodopsin and acid metarhodopsin with orange light produces a steady-state mixture containing roughly equal amounts of rhodopsin and acid metarhodopsin. This fact led them to the conclusion that the system involves no more than the reversible stereoisomerization of the chromophore in the reaction, rhodopsin $\rightleftharpoons$ metarhodopsin. Our previous paper[1] reported results clearly different from those of Hubbard and St. George[2].

There are two pathways in the regeneration of rhodopsin. One is photoregeneration, the direct back reaction from intermediates in the bleaching process by absorbing light; the other is the pathway in which opsin recombines with 11-*cis*-retinal which has been produced from all-*trans*-retinal by the action of either retinene isomerase[3] or retinochrome[4]. The latter mechanism can not be the cause in the phenomenon observed by us in squid because the regeneration of rhodopsin can occur even in the presence of hydroxylamine[1] which decomposes retinochrome[5].

Photoregeneration can be considered to be a reaction in the opposite direction to the bleaching process. It is well established that the bleaching of squid rhodopsin to metarhodopsin involves both photochemical and thermal reactions[6]. Accordingly, we might expect that the photoregeneration process could involve not only a photochemical reaction but also a thermal reaction, which would be due to conformational changes in the protein part of the pigment. However, no one has demonstrated a thermal process nor any intermediate states in the photoregeneration of squid rhodopsin or cattle rhodopsin.

In the present work we have studied the photoregeneration of squid rhodopsin in more detail and obtained the following results: The dark reaction of regeneration is scarcely observed in solutions made alkaline with sodium carbonate (pH 8.5–10.5). This agrees with the results reported by previous investigators[2]. In solutions made alkaline with borate–NaOH buffer (pH 8.65–10.65), the monomolecular reaction from the precursor to rhodopsin is easily observed. The precursor substance (λ_{max} 380 nm) is produced from acid metarhodopsin and converted to rhodopsin in the dark, and thus the substance is considered to be an intermediate in the photoregeneration of squid rhodopsin.

MATERIAL AND METHODS

Preparation of rhodopsin

Eye balls excised from squid (*Todarodes pacificus*) caught at Hachinohe on the northeast coast of Japan were frozen and brought to the laboratory in a dark ice-box. They were then kept in a freezer. The frozen eye balls were melted gradually at room temperature and the retinas dissected. The outer segments of the photoreceptors were removed by gently shaking the dissected retinas in 0.06 M phosphate buffer solution (pH 6.8), and pelleted by centrifugation (10000 rev./min, 30 min). The pellets were suspended in 40 % sucrose (pH 6.8) and rod outer segments were floated and isolated by repeated centrifugation. The isolated rod outer segments were then washed repeatedly with 0.067 M Na_2HPO_4 solution and distilled water to remove the screening pigment, ommochrome. Rhodopsin was extracted from the outer segments by mixing them with 2 % digitonin (0.01 M KH_2PO_4) and letting the mixture stand at 4 °C for 2 h. The extracts were then cleared by centrifugation (30000 rev./min, 60 min).

Ommochrome, which contaminated the rhodopsin solution, was completely eliminated by passing the extract through a DEAE-cellulose column. Salts in the rhodopsin solution obtained by this procedure were removed on a Sephadex G-25 column by eluting with an unbuffered solution of the desired digitonin concentration.

Spectroscopy and irradiation

Absorption spectra were measured with a Hitachi automatic spectrophotometer (EPS-3T), and the time courses of the absorbance changes at 480 nm and 380 nm were determined with a Hitachi-124 spectrophotometer. The temperature of the sample was kept constant by circulating water of the desired temperature through the cell holder. The temperature was monitored by immersing a Cu–Constantan thermocouple in the cell.

The sample was irradiated by light from a 500-W tungsten lamp which had passed through a glass filter transmitting wavelengths longer than 520 nm. This yellow light was focussed by an optical system and led to the sample cell through an optical glass fibre (5 mm in diameter). In order to determine absorbance at 480 nm and 380 nm during the irradiation, interference filters were used to isolate the spectrophotometer PM tube from the actinic light.

Other experimental details will be described together with experimental results.

RESULTS

Changes in the absorption spectrum of the irradiated rhodopsin

A rhodopsin solution containing 1.2% digitonin and 0.05 M borate–NaOH buffer (pH 10.2) was irradiated with yellow light ($\lambda > 520$ nm) for 5 min at 6 °C and absorption spectra were determined at frequent intervals after the irradiation. The results are shown in Fig. 1A. The absorbance at 480 nm is decreased by the irradiation and that at 380 nm is increased. In the dark, the absorption band with λ_{max} of 380 nm becomes smaller and the band at 480 nm reappears, showing the conversion of a substance with λ_{max} of 380 nm to the pigment with λ_{max} of 480 nm. The increase of the absorption band with λ_{max} of 480 nm is due to the formation of rhodopsin because circular dichroic absorbance (CD) also recovers[1]. There is an isosbestic point at 418 nm, indicating the direct conversion of a single substance to rhodopsin. Tentatively we will call the substance with λ_{max} of 380 nm P_{380}.

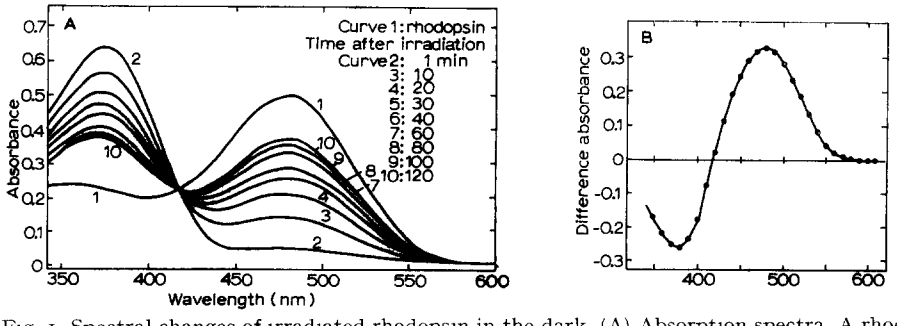


Fig. 1. Spectral changes of irradiated rhodopsin in the dark. (A) Absorption spectra. A rhodopsin solution containing 1.2% digitonin and 0.05 M borate–NaOH buffer (pH 10.2) (Curve 1) was irradiated with yellow light for 5 min at 6 °C, yielding a product with an absorption spectrum like alkaline metarhodopsin (Curve 2). In the dark, the absorption spectrum changes towards Curve 10. 90 s was needed for each scan from 340 to 600 nm. Times after irradiation are indicated in the figure. (B) Difference spectrum between Curve 2 and Curve 10

The difference spectrum between Curves 2 and 10 in Fig. 1A is shown in Fig. 1B. A negative peak is found at 380 nm and positive peak at 480 nm. The ratio of the difference at 380 nm to that at 480 nm is about 0.8.

Changes in absorbance at 480 nm and 380 nm in the light and the dark

The time courses of the changes in absorbance at 380 nm and 480 nm during and after the irradiation were determined at 5 °C, and the results are shown in Fig. 2A. The sample contains 0.1% digitonin and 0.1 M borate–NaOH buffer (pH 10.1). The absorbance at 480 nm is decreased by irradiation with yellow light and after the irradiation, it gradually increases in the dark until it is stable after 60 min. During irradiation, the absorbance at 380 nm rapidly increases and then decreases a little to reach the steady state. After cessation of irradiation, the decrease in absorbance at 380 nm was antiparallel in time course to the increase in absorbance at 480 nm. When the logarithm of absorbance is plotted against the time after irradiation, the points lie on straight lines as shown in Fig. 2B, indicating a monomolecular reaction. From the result in Fig. 2B, the reaction constant (k) of the monomolecular reaction was calculated to be $1.18 \cdot 10^{-3}$ s^{-1}. The ratio of $\Delta A_{380\,nm}$ to $\Delta A_{480\,nm}$ is 0.78. If alkaline metarhodopsin has been converted to rhodopsin, the ratio should be about 1.25 (refs 2 and 7). Accordingly we estimate ε_{max} of P_{380} to be about 3/5 of the value for alkaline metarhodopsin. The fact that the absorbance at 380 nm first increased and then decreased a little during the irradiation (Fig. 2A) can be explained if alkaline metarhodopsin is first produced and then is converted to P_{380} whose ε_{max} is much lower.

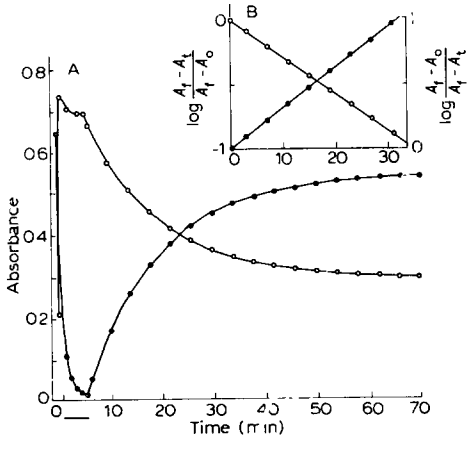


Fig. 2. Time courses of absorbance in the light and the dark. (A) Time courses of absorbance at 480 nm and 380 nm. ○—○, absorbance at 380 nm; ●—●, absorbance at 480 nm. The irradiation period (5 min) is underlined in the figure. The temperature of the sample was kept at 5 °C. Detergent–buffer system: 0.1 M borate–NaOH buffer (pH 10.1), 0.1% digitonin. (B) Kinetics of the changes in absorbances in the dark. The values of $\log A_f - A_t / A_f - A_0$ (at 380 nm) and $\log A_f - A_0 / A_f - A_t$ (at 480 nm) were plotted against the time after the irradiation as open circles and solid circles, respectively. A_0 and A_f indicate initial (light off) and final (completion of reaction) absorbances, respectively. A_t is the absorbance at time t after the irradiation. The reaction constant (k) can be obtained from the following equation:

$$k = \frac{2.303}{t} \log \frac{A_f - A_0}{A_f - A_t}$$

Effect of pH on the velocity of the regeneration

Effect of pH on the reaction velocity of the conversion of P_{380} to rhodopsin was examined. The time course of the change in absorbance at 480 nm was determined at various pH values which were adjusted by adding 0.05 M borate–NaOH or borate–HCl buffers at 5 °C. The results are shown in Fig. 3A. Fig. 3A indicates that the reaction is first order at any pH examined here. The reaction constants were calculated and are plotted on a logarithmic scale against pH in Fig. 3B. A linear correlation is found between log k and pH which can be expressed as

$$\log k = -0.67\,(\mathrm{pH}) + 4.17 \qquad (1)$$

From Eqn 1, the reaction constant at pH 7.0 is expected to be $3.56 \cdot 10^{-1}\ s^{-1}$.

Effect of temperature

The amount of rhodopsin regenerated was dependent on the temperature of the rhodopsin solution during the irradiation period[1]. This fact suggests that the reaction velocity of $P_{380} \rightarrow$ rhodopsin strongly depends on the temperature and that the higher the temperature, the less P_{380} accumulates during irradiation.

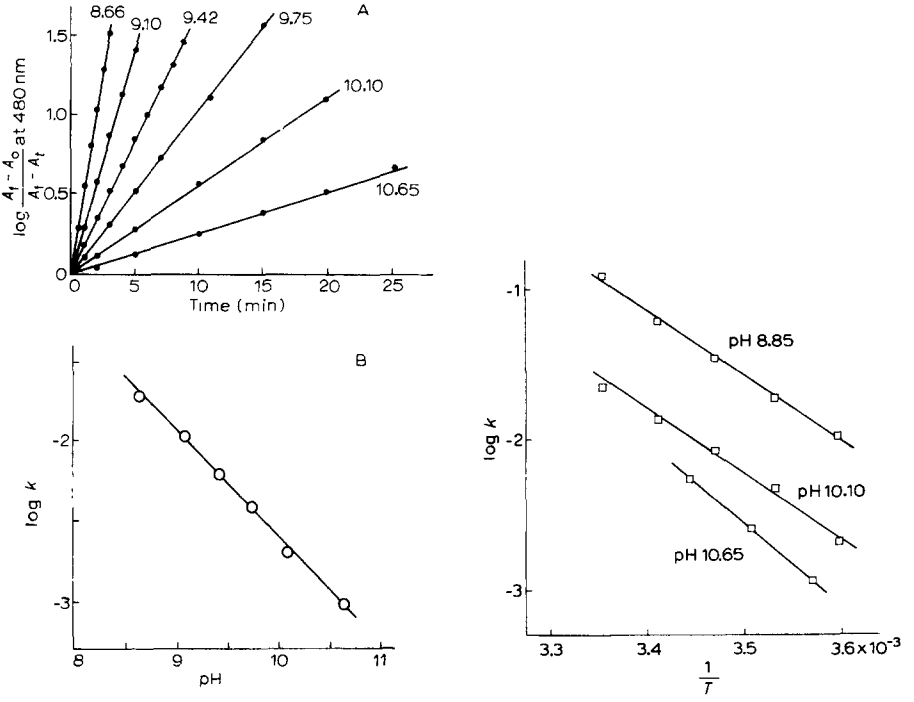


Fig. 3. Effect of pH on the reaction velocity of the conversion of P_{380} to rhodopsin. (A) Kinetics of the formation of rhodopsin from P_{380} in the dark The pH was adjusted with 0.05 M borate–NaOH or borate–HCl buffer and the temperature of the sample was kept at 5 °C. Concentration of digitonin is 0.1%. (B) Plot of logarithm of the reaction constant against pH of the solution.

Fig. 4. Arrhenius plots for the conversion of P_{380} to rhodopsin. Rhodopsin solutions adjusted to pH 8.85, 10.1 and 10.65 with 0.05 M borate–NaOH or borate–HCl buffer were irradiated with yellow light for 5 min and the changes in absorbance at 480 nm in the dark were recorded at various temperatures. Reaction constants were calculated as in Fig. 2

The effect of temperature on the reaction velocity was examined at pH 8.85, 10.1 and 10.65. The results are shown in Fig. 4. There is a linear relationship between log k and $1/T$ at each pH. From the results in Fig. 4, the activation energy (E_a), as calculated by the Arrhenius equation, is 20.0, 19.7 and 24.9 kcal/mole at pH 8.85, 10.1 and 10.65, respectively. These results indicate that the reaction mechanism of the transformation is the same at any of the temperatures and pH values examined here.

Effect of composition of the solvent

The reaction velocity of the conversion of P_{380} to rhodopsin is influenced by the composition of the solvent. Reaction constants were determined in 0.1 % digitonin at pH 9.9, 5 °C with various concentrations of borate–NaOH buffer. The reaction constant decreased with increasing buffer concentration as is shown in Table I.

Digitonin, also, affects the velocity of the conversion of P_{380} to rhodopsin. Reaction constants were determined at various concentrations of digitonin at pH 10.0 (0.05 M borate–NaOH buffer) and 10 °C. The results are shown in Table II. The value of the reaction constant is decreased as the concentration of digitonin increases until it reaches a constant value at concentrations > 1 %.

When rhodopsin solutions containing 0.1 % digitonin were made alkaline with 0.1 M Na_2CO_3–$NaHCO_3$ buffer (pH 8.5–10.5) and irradiated with yellow light at 5 °C, only small amounts of rhodopsin (less than 10 % of initial rhodopsin) could be regenerated in the dark. Thus almost all rhodopsin could be converted to alkaline metarhodopsin at pH 10.5. Addition of borax to the alkaline metarhodopsin thus obtained did not in itself induce the regeneration of rhodopsin in the dark. However, after irradia-

TABLE I

EFFECT OF BUFFER CONCENTRATION ON REACTION CONSTANT DETERMINED AT pH 9.9 AND 5 °C IN 0.1 % DIGITONIN SOLUTION

Concentration (*M*)	k (s^{-1}) × 10^3
0.001	5.02
0.01	4.01
0.03	2.68
0.1	1.81

TABLE II

EFFECT OF DIGITONIN CONCENTRATION ON REACTION CONSTANT DETERMINED AT pH 10.0, 10 °C AND 0.05 M BORATE–NaOH BUFFER

Concentration (%)	k (s^{-1}) × 10^3
0 025	5 41
0.075	4.22
0.125	3.52
0.525	2.01
1.025	1.63
2.525	1.43

tion of the solution with yellow light, rhodopsin was regenerated in the same way as in a solution originally containing borax. These facts strongly suggest that bicarbonate/carbonate does not inhibit the regeneration but that a specific action of borate ions on opsin must be important in the production of P_{380}.

Production of P_{380} during the irradiation

From the transient increase of absorbance at 380 nm during the irradiation as shown in Fig. 2A, it was suggested that the formation of alkaline metarhodopsin is followed by the production of P_{380}. How does the amount of P_{380} change under irradiation with yellow light ($\lambda > 520$ nm)? This question was examined using solutions of rhodopsin in 0.1 % digitonin at 0.1 M borate–NaOH buffer (pH 10.1) and 6 °C. The time courses of the changes in absorbance at 480 nm of the rhodopsin solutions irradiated for 10 s and 90 s are shown in Figs 5A and B, respectively. When rhodopsin was irradiated for 10 s, much less rhodopsin was regenerated than when the rhodopsin was irradiated for 90 s. This result indicates that the production of P_{380}, the precursor of rhodopsin, is dependent on the duration of irradiation. The amounts of rhodopsin, metarhodopsin and P_{380} are indicated by a, b and c, respectively, in Figs 5A and 5B. In this case, the amount of acid metarhodopsin which contributes to the absorbance at 480 nm is neglected because the concentration of acid metarhodopsin is only one-tenth of alkaline metarhodopsin at pH 10.1 (pK of the equilibrium, acid metarhodopsin $\rightleftharpoons$ alkaline metarhopsin, is 9.1[7]). Thus we could determine the time courses

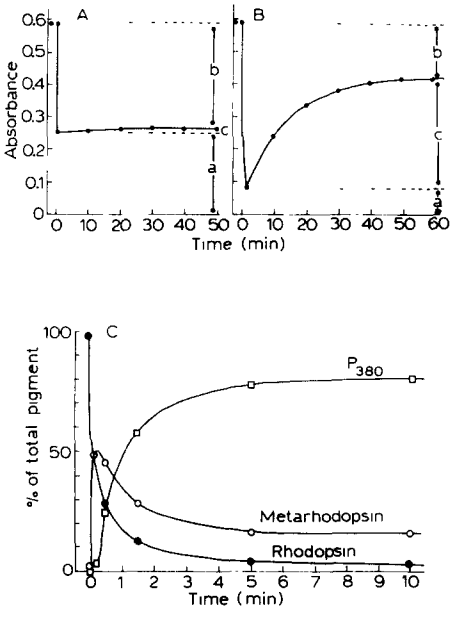


Fig. 5. Time courses of concentrations of rhodopsin, metarhodopsin and P_{380} during the irradiation. (A and B) Time courses of absorbance at 480 nm in the light and the dark. Rhodopsin solution is irradiated for 10 s (A) and for 90 s (B) at 6 °C with yellow light. (C) Time courses of the concentrations of rhodopsin, metarhodopsin and P_{380} under the irradiation with yellow light. Concentration of each substance is expressed as per cent of the total pigment in the mixture. Composition of the suspension medium: 0.1 M borate–NaOH buffer (pH 10.1), 0.1% digitonin.

of the amounts of rhodopsin, metarhodopsin and P_{380} during the irradiation, and the results are shown in Fig. 5C.

Rhodopsin is simply decreased by light. Metarhodopsin rapidly increases to reach a maximum and then gradually decreases. After metarhodopsin reaches its maximum, P_{380} increases and finally arrives at a plateau of about 80% of total pigments. The results in Fig. 5C indicate that rhodopsin is converted to metarhodopsin which is then convented to P_{380} by light. Because the irradiating light contains only wavelengths longer than 520 nm, which can not be absorbed by alkaline metarhodopsin, P_{380} must be produced by light absorbed by acid metarhodopsin. Presumably since acid metarhodopsin is concerned in this conversion, the equilibrium between acid and alkaline metarhodopsin provides new acid metarhodopsin from alkaline metarhodopsin.

Configuration of the chromophore of P_{380}

The fact that P_{380} is produced from acid metarhodopsin by light suggests that the chromophore of P_{380} may be in the 11-*cis*-configuration. If this is so, the amount of rhodopsin regenerated in the dark should be reduced by reirradiation of P_{380} with near ultraviolet light. In order to elucidate the configuration of the chromophore of P_{380}, the following experiments were carried out.

The rhodopsin solution containing 0.1% digitonin and 0.1 M borate–NaOH buffer (pH 10.1) was irradiated with yellow light for 5 min at 5 °C. The irradiated rhodopsin was then allowed to stand in the dark for 60 min at 10 °C to regenerate rhodopsin. The regenerated rhodopsin was reirradiated with yellow light for 5 min at 5 °C and immediately frozen in liquid nitrogen to stop the conversion of P_{380} to rhodopsin. The frozen sample was irradiated with light through an interference filter transmitting near ultraviolet light (λ_{max} 377 nm). It then was warmed to 10 °C and left to stand for 60 min in the dark.

The results of this experiment are shown in Fig. 6. Curve 1 is the absorption spectrum of rhodopsin before irradiation at pH 10.1 and Curve 2 is that of the regen-

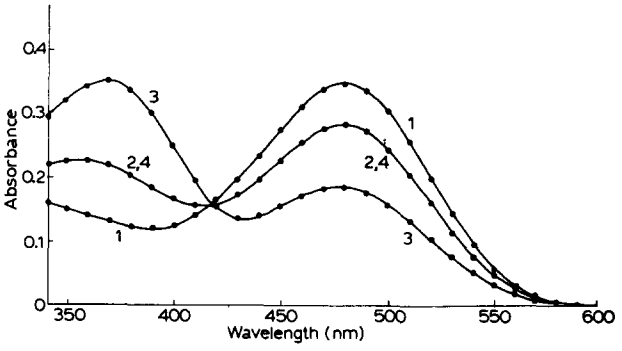


Fig. 6. Effect of irradiation with near ultraviolet light on the regeneration. Curve 1, rhodopsin in the solvent containing 0.1 M borate–NaOH buffer (pH 10.1) and 0.1% digitonin; Curve 2, regenerated rhodopsin after irradiation with yellow light for 5 min at 5 °C; Curve 3, regenerated rhodopsin after irradiation of the sample shown in Curve 2 with yellow light for 5 min at 5 °C and then with near ultraviolet light for 2 min at liquid-nitrogen temperature; Curve 4, regenerated rhodopsin after irradiation of the sample of Curve 3 with yellow light for 5 min at 5 °C. Further explanation is in the text.

erated rhodopsin after the first irradiation with yellow light. Since about 80 % of the original rhodopsin was regenerated, then about 80 % of the initial rhodopsin must have been converted to P_{380} by the irradiation. When the solution of Curve 2 is reirradiated with yellow light for 5 min at 5 °C and then with near ultraviolet light for 2 min at liquid-nitrogen temperature, the amount of rhodopsin regenerated in the dark is reduced to about 60 % of initial rhodopsin (Curve 3). This result can be explained if the chromophore of P_{380} is in the 11-*cis*-configuration and is reisomerized to the all-*trans*-form by irradiation with near ultraviolet light. When the sample of Curve 3 is irradiated again with yellow light at 5 °C for 5 min and allowed to stand for 60 min at 10 °C in the dark, about 80 % of the initial rhodopsin is regenerated and the solution shows Curve 4 which is identical with Curve 2. We also confirmed that when the rhodopsin solution irradiated with yellow light was frozen in liquid nitrogen and then warmed to 10 °C, the same amount of rhodopsin was regenerated as in the case without freezing. This indicates that this freezing procedure does not affect the regeneration of rhodopsin.

When the irradiation of the sample containing predominantly P_{380} with near ultraviolet light is prolonged, the regenerated pigment shows a λ_{max} less than 480 nm and higher absorbance at the λ_{max} than that of the sample irradiated for 2 min. This can probably be ascribed to the generation of isorhodopsin from 9-*cis*-retinal produced by the prolonged irradiation with near ultraviolet light at liquid-nitrogen temperature.

We could also confirm that the chromophore of P_{380} was in the 11-*cis*-configuration from the following experiment. A rhodopsin solution was irradiated with yellow light and the resulting steady state mixture containing more than 80 % P_{380} was denatured with NaOH. The pH of the denatured preparation was adjusted to 7.0 with HCl. The denatured P_{380} was mixed with cattle opsin and incubated at 20 °C for 2 h in the dark. The absorption band at 498 nm gradually increased in the dark. The difference spectrum before and after irradiation in the presence of NH_2OH indicated the curve characteristic of the decomposition of cattle rhodopsin and formation of retinal oxime. The amount of cattle rhodopsin regenerated was 77 % of that of the rhodopsin regenerated from cattle opsin and squid rhodopsin denatured by the same procedure. Only trace amounts of cattle rhodopsin were regenerated when cattle opsin was mixed with the denatured squid metarhodopsin.

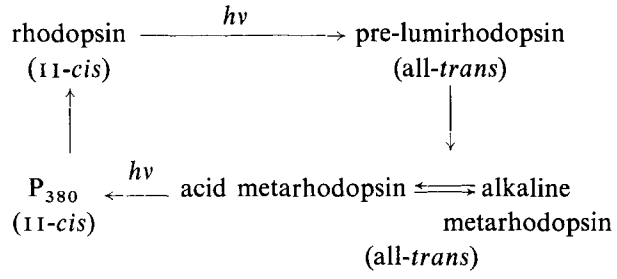
DISCUSSION

From the results in Fig. 5, the following pathway can be proposed for the formation of P_{380}.

$$\text{Rhodopsin} \rightarrow \text{metarhodopsin} \rightarrow P_{380}$$

In the present experiments, the irradiation is carried out with light containing only wavelengths longer than 520 nm which can not be absorbed by alkaline metarhodopsin, and accordingly P_{380} must be produced from acid metarhodopsin which can absorb this yellow light.

Now, the squid rhodopsin cycle under irradiation with yellow light can be illustrated as follows:



The bleaching process involves photochemical (rhodopsin → pre-lumirhodopsin) and thermal reactions (pre-lumirhodopsin → metarhodopsin) as clarified by work at low temperatures[6,8–10]. The photoregeneration process also involves both photochemical and thermal reactions, the directions of which must be the reverse of those in the bleaching process. P_{380}, with 11-*cis*-retinal as chromophore, is produced from metarhodopsin by light and is converted to rhodopsin in the dark. Thus P_{380} is nothing but an intermediate in the photoregeneration process of squid rhodopsin.

Changes in the ultraviolet absorption spectrum at 235 nm during the bleaching process have been reported for squid rhodopsin[11] as well as for cattle rhodopsin[12], indicating the conformational changes of opsin in the transformation from rhodopsin to metarhodopsin. From the observation that the reaction constant for the conversion of P_{380} to rhodopsin is dependent not only on pH and temperature but also on buffer and digitonin concentrations, it is reasonable to suppose that the reaction of P_{380} → rhodopsin is accompanied by a conformational change in opsin. The conformation of opsin must fit 11-*cis*-retinal in rhodopsin and all-*trans*-retinal in metarhodopsin (*cf.* ref. 2). When all-*trans*-retinal in metarhodopsin is isomerized to the 11-*cis*-form, opsin may change its conformation to fit 11-*cis*-retinal again. The pH dependence of the velocity of the P_{380} → rhodopsin transition (Fig. 3) suggests that P_{380} requires protons in its conversion to rhodopsin.

In the conversion of P_{380} → rhodopsin, the λ_{max} is red shifted about 100 nm, which is probably due to the protonation of the Schiff base linkage between the chromophore and the protein. However, this reaction can proceed even at pH 10.65 under conditions where the protonated Schiff base in free solution scarcely exists (*cf.* ref. 13). According to our theory of the color of the visual pigment[14], the red shift of the λ_{max} can be explained if there is a proton donor in the vicinity of the Schiff base in opsin and this donor is in the proper position to donate a proton to the Schiff base when the conformation of opsin changes to fit 11-*cis*-retinal in the change from P_{380} to rhodopsin.

The role of borate ions in the regeneration process is unclear. Studies on the CD spectrum of rhodopsin have shown that the conformation of the rhodopsin molecule is sensitive to its environment[15,16]. It might be that the rhodopsin molecule is kept in the special conformation which favors the production of P_{380} by interactions with borate ions.

The phenomena reported in the present paper can be observed only under alkaline conditions and not under neutral conditions because the velocity of the conversion of P_{380} to rhodopsin is strongly dependent on the pH (see Fig. 3) and is too fast to be observed under neutral conditions. Recently, in the investigation of the early receptor potential of the octopus retina, Tsukahara[17] obtained an important

result indicating the production of an intermediate in the photoregeneration of rhodopsin by yellow light and its conversion to rhodopsin in the dark. It is not yet possible to say whether the pathway of photoregeneration demonstrated here occurs in the living retina or whether any other reaction takes place. Further studies are needed to answer this question.

The present work demonstrates that a new substance, P_{380}, is an intermediate in the photoregeneration process of squid rhodopsin. The transformation from P_{380} to rhodopsin is a thermal process involving a conformational change in opsin fitting to 11-*cis*-retinal and protonation of the Schiff base, the primary linkage of the chromophore to opsin.

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