Biochimica et Biophysica Acta, 502 (1978) 495—506

BBA 47504

KINETIC STUDY OF PHOTOREGENERATION PROCESS OF DIGITONIN-SOLUBILIZED SQUID RHODOPSIN

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(Received April 26th, 1977)
(Revised manuscript received December 16th, 1977)

Summary

In the photoregeneration process of squid rhodopsin, an intermediate has been found at neutral pH values (phosphate buffer) with a flash light ($\lambda > 540$ nm). An intermediate R430, with the 11-*cis* retinal as chromophore, is produced from metarhodopsin in light and is converted to rhodopsin through the processes R430 → P380 and P380 → rhodopsin. The pH dependence of the velocity of the conversions suggests that processes R430 → P380 and P380 → rhodopsin involve a protolytic reaction and that the ionized group is a histidine residue of opsin. Kinetic parameters show that the largest conformational change in opsin occurs in the conversion of R430 → P380.

Introduction

Light absorption by the chromophore of rhodopsin results in its isomerization from the 11-*cis* to the all-*trans* form, and this triggers a series of reactions leading to visual excitation. Regeneration of rhodopsin, which involves reisomerization of the all-*trans* retinal thus formed to the 11-*cis* isomer, is an essential process for maintaining unimpaired vision. In recent years, several authors [1—9] have investigated the mechanisms for the regeneration of rhodopsin. One is photoregeneration, either from different thermal intermediates in the bleaching process [4,5] or from the final photoproduct [7—10]; the other is a dark process in which the 11-*cis* retinal is produced from the all-*trans* retinal by the action of "retinal isomerase" and reacts with opsin [11—14]. Current evidence [11—13] overwhelmingly indicates that the 9-*cis* isomer is formed in the dark in the retina. Shichi and Somers [15,16] showed that the photoisomerization of all-*trans* retinal to the 11-*cis* isomer proceeded efficiently when the retinal was in the form of a protonated retinylidene phosphatidylethanolamine. They proposed a rhodopsin regeneration cycle involving the retinylidene com-

plex. It has been shown that rhodopsin of cephalopod regenerates from its final product, metarhodopsin, in light [6,8—10]. These facts suggest that there is a photochemical cycle of rhodopsin which is distinct from the so-called visual cycle.

Photoregeneration can be considered to be a reversal of the bleaching process. It is well established that the bleaching process of rhodopsin involves both photochemical and thermal reactions [17]. It is therefore expected that the photoregeneration process might involve not only a photochemical reaction but also a thermal reaction, which would accompany conformational changes of opsin protein. For the study of the regeneration process, cephalopod rhodopsin is very useful because metarhodopsin, the final decomposition product of this pigment, is stable below 15°C and can be reversed to rhodopsin in light [6].

Recently, Kito et al. [8] found P380 as an intermediate in the photoregeneration process of squid rhodopsin. However, P380 is produced in the presence of $B_4O_7^{2-}$ under the continuous irradiation of yellow light. In this paper we first attempt to elucidate whether or not P380 exists in the absence of $B_4O_7^{2-}$ on irradiation with a pulse light. As shown by many authors [17], there are several steps in the photobleaching process of rhodopsin. Accordingly, we can expect that some intermediate steps may proceed before the conversion of P380 to rhodopsin in the regeneration of rhodopsin. Secondly, we try to identify and characterize such intermediates of the photoregeneration process.

Experimental

Materials

Squids (Surumeika, *Todarodes pacificus)* were caught at Horomui on the Shakotan peninsula, in western Hokkaido, Japan. They were collected and decapitated at once in dim light, and their eye balls, kept dark at —80°C, were brought to the laboratory and stored in a freezer.

Squid rhodopsin was prepared by the method described by Suzuki et al. [19]. When the eye-cup was gently shaken upside-down in 67 mM phosphate buffer solution at pH 6.8, outer segments sank with fragments of black pigment. The outer segments were purified by repeated sucrose gradient centrifugation. The outer segments containing sucrose (density 1.20 g/ml) were layered at the bottom of a discontinuous sucrose gradient in 10 mM phosphate buffer (pH 6.8) containing 1 mM $MgCl_2$ and 10 mM NaCl. The gradient was formed by layering 2 ml sucrose solutions of different densities (1.15, 1.13 and 1.11 g/ml). After centrifugation at 21 000 $\times g$ for 2 h at 5°C, a dense orange band appeared at the 1.13—1.15 g/ml interface, which was collected with a syringe with a long needle (No. 18). This discontinuous gradient centrifugation was repeated twice. The isolated outer segments were then washed repeatedly with distilled water, 10 mM KH_2PO_4, 100 mM Na_2HPO_4, 67 mM phosphate buffer (pH 6.8) and finally with 0.01% digitonin solution by centrifugation. Rhodopsin was extracted from the outer segments with 2% digitonin in 100 mM phosphate buffer at 4°C for 2 h. The extracts were then cleared by centrifugation (25 000 $\times g$, 60 min). The rhodopsin extracts were purified on a DEAE-cellulose column [19]. Ommochrome, which was identified as ommin [20], did not contaminate the preparation in view of the absence of a band at the top of the

column. A hydroxylamine assay [20] showed that the preparation contained scarcely any retinochrome.

A metarhodopsin solution was obtained as follows. When a rhodopsin solution in phosphate buffer (100 mM, pH 6.8) containing 2% digitonin was made alkaline (pH 10.5) with a grain of Na_2CO_3 and irradiated with yellow light ($\lambda >$ 500 nm) from a 500 W Xe-short arc lamp at 5°C, almost all rhodopsin was converted to alkaline metarhodopsin. The buffer in the alkaline metarhodopsin solutions was repeatedly exchanged with 0.4% digitonin-containing 100 mM phosphate buffer at an appropriate pH using an Amicon Centriflo membrane ultrafilter Type CF25.

Methods

Transient absorption spectra were studied using a rapid-scan spectrophotometer (UNION RA-1300) [21] equipped with a flash apparatus. After irradiation of a flash light, the spectral pattern was measured by means of an image decector with a maximum speed of 30 nm/ms and was memorized by a transient recorder. The device contains five 512-word memories with 8 bit resolution. An analog replica of the original could be recorded on an X-Y recorder as a spectral pattern of absorbance or time-resolved difference spectrum. An image of panchromatic light from a halogen lamp entered the sample cell and the light was dispersed by grating and received by the image decector tube. In order to protect the sample from bleaching, a particular wavelength was selected using glass filters (Toshiba V-B46, VY-40 and Hoya thermal filter). However, the absorbance of the main band was changed by monitoring light for more than 500 ms after opening the shutter (0.005 ΔA at 1 s later). In the observation lasting more than 500 ms, the shutter was opened only when the absorption spectrum was recorded.

A highly sensitive single flash apparatus was assembled to observe the transient changes in absorbance of the intermediates. The monitoring source was rendered monochromatic by means of a monochromator, which was placed in front of the sample cell, and its intensity was sufficiently low so that no bleaching of the sample was observed over a period of minutes. A gradient interference filter (Optical Coating Laboratory) was placed in front of the photomultiplier housing to protect the phototube against scattered light from the flash.

The flash lamp (Xe, approx. 50 J) had a half duration of approx. 20 μs. The beam was focused on the sample cell using an elliptical mirror, a thermal filter (Hoya) and an orange glass-filter (Toshiba VO-54).

Measurements were made at temperatures between 1.5 and 13.4°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 (Yamato BL-31) was circulated in the inner jacket. The temperature within the sample cell was measured with a thermocouple.

The reaction was recorded by means of a kinetic data processor (UNION RS-450). This contained a transient recorder which possessed 8 bit data (256 data unit) resolution and 8 bit time (256 channel) resolution. With this processor, data fluctuations could be minimized with accumulation of data up to 255 times. The kinetic record which is digitized and stored by the transient recorder can be recorded on an X-Y recorder, or displayed on an oscilloscope and photographically recorded.

Results

Absorbance change with time of the metarhodopsin solution with flashing

The intermediate in the photoregeneration process of rhodopsin has been studied by Kito et al. [8] and they showed an intermediate P380 having an absorption maximum of 380 nm.

P380 was observed when rhodopsin was made alkaline with B_4O_7/NaOH and irradiated continuously with yellow light ($\lambda > 520$ nm). It is interesting whether P380 can be observed or not when a metarhodopsin solution at neutral pH without $B_4O_7^{2-}$ was irradiated with a pulse flash. Under these conditions accumulation of P380 could scarcely be observed.

In order to study the intermediate processes in the photoconversion of metarhodopsin to rhodopsin, transient changes in absorbance were recorded after an exciting flash at various wavelengths (360—650 nm). Fig. 1 shows the typical patterns of the transient-memory recordings for the metarhodopsin solution in 0.4% digitonin and 100 mM phosphate buffer (pH 8.4) at 5.5°C. A kinetic curve in Fig. 1A shows that a rapid decrease and a subsequent slower increase in the absorbance at 480 nm were observed. This suggests that metarhodopsin disappeared during the flash, and rhodopsin was regenerated. On the other hand, the absorbance at 380 nm showed a rapid increase and a subsequent slower decrease. The slower decrease in absorbance might reflect the decay of P380. This assumption may be confirmed by the fact that the rate of decrease in the absorbance at 380 nm was similar to that of the absorbance increase at 480 nm. A hump observed in the early stage of the absorbance changes at 480 and 380 nm suggested that a precursor of P380 might exist in

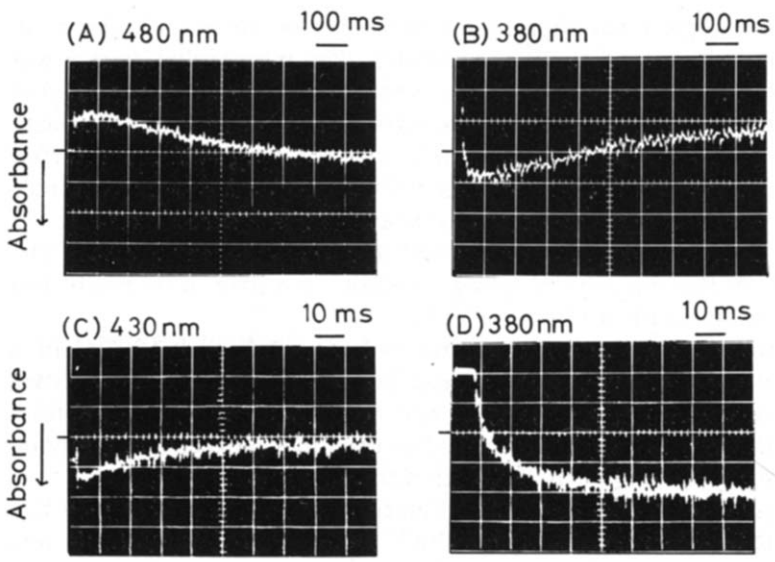


Fig. 1. Transient-memory recording curves obtained in typical photolysis experiments. A metarhodopsin solution containing 0.4% digitonin and 0.1 M phosphate buffer (pH 8.4) was irradiated with a flash of orange light ($\lambda > 540$ nm) at 5.5°C. Absorbance decreases are recorded upwards. The initial absorbance is indicated by the horizontal bar on the left side in each picture.

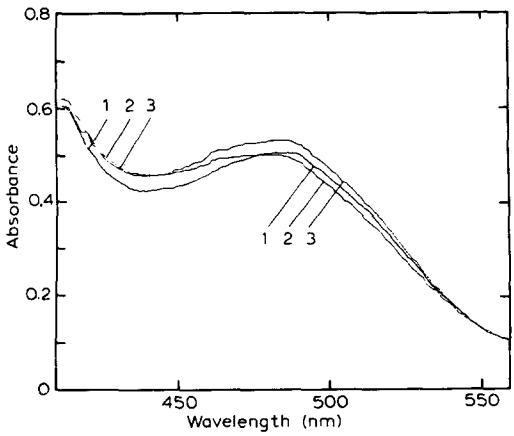


Fig. 2. Transient changes in absorption spectra obtained by the rapid-scanning flash photolysis technique. Scanning speed was 30 nm/ms. Curve 1: absorption spectrum of the metarhodopsin solution in 0.4% digitonin/0.1 M phosphate buffer (pH 8.3) at 4.3°C before flashing. The scanning of curves 2 and 3 was started at 0 ms and 60 s, respectively, after the light flash ($\lambda > 540$ nm).

the photoregeneration process of rhodopsin. The increase in the absorbance at 380 nm in Fig. 1D corresponds to the earlier stage in Fig. 1B and shows the formation of P380. Logarithmic plots of the reaction curve in Fig. 1D shows that the curve was not first order, but was consistent with a set of independent first order reactions. Further, we found a sudden increase and slower decrease in absorbance around 430 nm. The rate of decrease in the absorbance at 430 nm corresponded to the rate for the slower phase in the reaction curve at 380 nm. This suggests the existence of an intermediate having a band around 430 nm in the difference spectrum, and which is a precursor of P380. The faster phase in the reaction curve at 380 nm and the sudden decrease in absorbance at 430 nm also suggest the existence of an intermediate. The spectrum of the intermediate having the shortest life time could not be observed, so we have named this intermediate RX. The intermediate following RX has been named R430, since it had a maximum absorbance in the difference spectrum around 430 nm. R430 may be a precursor of P380 since the rate of the decrease in absorbance with time around 430 nm corresponded to that of the increase in absorbance with time at 380 nm. The last intermediate was P380 from which rhodopsin is regenerated.

Transient spectral changes in metarhodopsin after a flash

Fig. 2 shows the results of transient spectral changes in the photoregeneration process from metarhodopsin to rhodopsin observed with the rapid-scan spectrophotometer equipped with a flash apparatus (scanning speed constant at 30 nm/ms). Curve 1 in Fig. 2 revealed an absorption spectrum of the metarhodopsin solution containing 0.4% digitonin and 100 mM phosphate buffer (pH 8.3) before flashing. Immediately after the flash with orange light ($\lambda > 540$ nm), the absorbance at a longer wavelength of the absorption band fell and its absorbance at a shorter wavelength rose (curve 2 in Fig. 2). Finally (60 s later), the absorption band around 480 nm rose and the band around 430 nm appeared slightly.

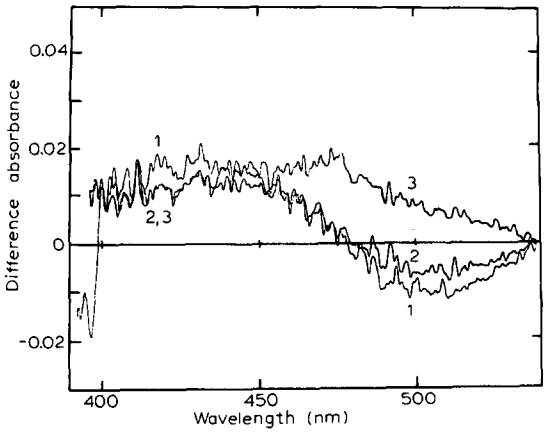


Fig. 3. Time resolution difference spectrum obtained by the rapid-scanning flash photolysis technique. Scanning speed was 30 nm/ms. The spectrum of the metarhodopsin solution before flashing served as reference. The scanning of the spectral curves 1, 2 and 3 started at 0 ms, 20 ms and 60 s after the flash. The conditions are the same as those in Fig. 2.

The sample used in this experiment contained acid metarhodopsin and alkaline metarhodopsin. The absorption band around 480 nm in curve 2 was due only to acid metarhodopsin, though rhodopsin had a similar absorption band. The results suggest that the decrease in absorbance around 480 nm, which was observed immediately after the flash, was due to decay of acid metarhodopsin. Reappearance of the band 60 s after flashing was due to rhodopsin, which regenerated from metarhodopsin in light. A slight increase in absorbance around 430 nm suggests the existence of an intermediate in the photoregenerate sequence of rhodopsin. To clarify the transient changes in absorption spectra, we observed the time-resolved difference spectrum obtained with the analog device by subtracting the absorbance at an appropriate time after the flash from the corresponding absorbance before flashing. The results are illustrated in Fig. 3. Curve 1 in this figure is the difference spectrum obtained immediately after the flash with orange light ($\lambda > 540$ nm) at pH 8.3 at 4.3°C, and the curve corresponds to the difference between curve 1 and curve 2 in Fig. 2. The results gave a negative band around 510 nm and a positive band around 430 nm. Curve 3 in Fig. 2 is the difference spectrum observed 20 ms after the flash. Both the negative and positive bands decreased in intensity compared with those in curve 1. Curve 3 was obtained 60 s later. A negative band around 510 nm in Curve 1 and curve 2 increased in absorbance with time and, finally, a positive band around 470 nm appeared. On the other hand, the positive band around 430 nm was not altered at 20 ms and 60 s. The results suggest that an intermediate having a band around 430 nm in the difference spectrum occurs in the regeneration process of rhodopsin.

Effect of temperature

The effect of the temperature on the rate of the thermal conversions of R430 → P380, and P380 → rhodopsin in the photoregeneration process of rhodopsin was studied at pH 8.3. The temperature range employed was 1.5—

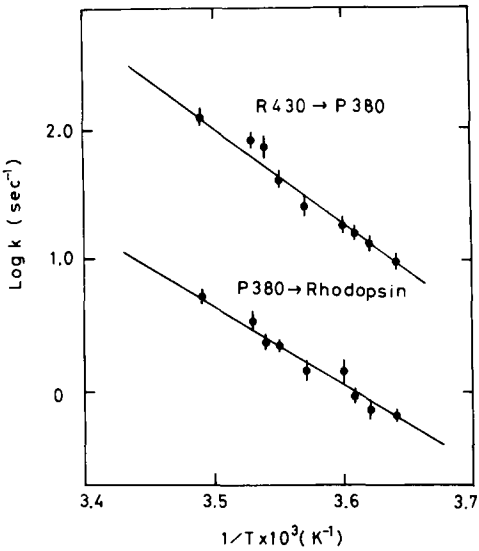


Fig. 4. Arrhenius plots for the interconversions of the intermediates. The R430 → P380 and P380 → rhodopsin conversions were observed as the absorbance changes at 430 and 480 nm. Detergent buffer system: 0.1 M phosphate buffer (pH 8.3)/0.4% digitonin.

13.4°C. Squid metarhodopsin is stable below 15°C. Arrhenius plots of the rates are illustrated in Fig. 4. The values of the enthalpy and entropy of activation were determined at pH 8.4 in 0.4% digitonin solution and are, for the process R430 → P380, 35.6 kcal/mol and 74.0 cal/deg. per mol and for the process P380 → rhodopsin, 26.8 kcal/mol and 36.9 cal/deg. per mol, respectively.

It is generally assumed that the major conformational changes in proteins are accompanied by large positive enthalpies and entropies of activation. Suzuki et al. [10] suggested in their ultraviolet absorption and CD studies that the major conformational change in opsin takes place in the conversion of metarhodopsin to P380 and only a minor one in the conversion of P380 to rhodopsin. As shown above, the conversion from R430 to P380 is accompanied by the largest enthalpy and entropy of activation. Therefore, the largest conformational changes of opsin appear to take place in this transformation.

Effect of pH on the velocity of the intermediate processes in regeneration

The pH dependence of kinetic parameters associated with opsin conformational changes can provide information about ionizable groups on rhodopsin in its regeneration process as well as give a clue to understanding the molecular basis of the change. The velocities of the conversions of R430 → P380 and P380 → rhodopsin in the regeneration process were examined at various pH values. The pH was adjusted with 100 mM phosphate buffer containing 0.4% digitonin at 5.5°C, and the results are plotted against the pH values in Figs. 5 and 6. The results suggest that the velocities of the conversions of R430 → P380 and P380 → rhodopsin were accelerated with a decrease in the pH. The following mechanism is consistent with these results:

$$R_1 + H^+ \overset{K_1}{\rightleftharpoons} R_1H \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} R_2H \qquad (1)$$

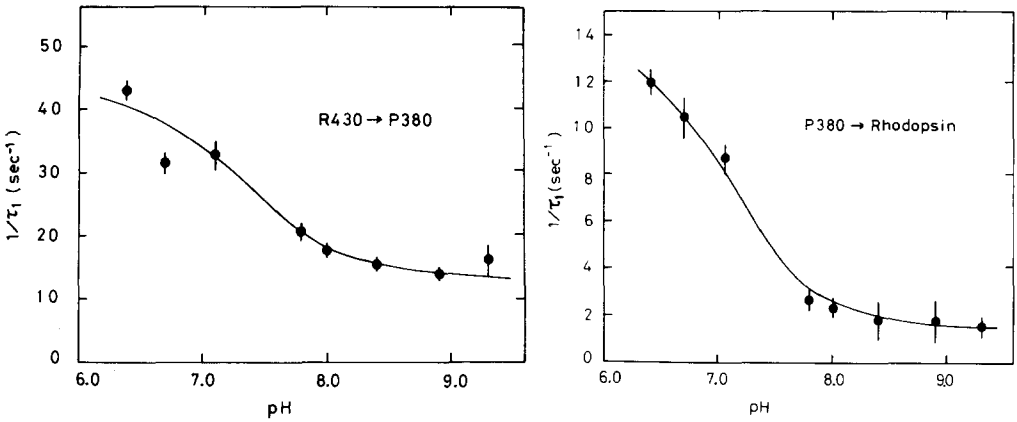


Fig. 5. Dependence of $1/\tau_1$, for the conversion of R430 to P380 against pH of the solution with 100 mM phosphate buffer/0.4% digitonin at 5.5°C. The solid line is the theoretical curve that was obtained by the least-squares treatment described in the text.

Fig. 6. Dependence of $1/\tau_1$ on pH for the conversion of P380 to rhodopsin. The conditions are the same as those in Fig. 5. The solid line is the theoretical curve that was obtained by the least-squares treatment described in the text.

where R_1H and R_2H represent two isomers of the intermediate. When the rate of equilibration between R_1 and R_1H is rapid compared to that between R_1H and R_2H, the expression for the slow relaxation time can be written as follows [22]:

$$1/\tau_1 = k_{21} + k_{12}/(1 + K_1/[H^+]) \quad (2)$$

The theoretical curves were obtained by a least squares analysis of Eqn. 2, where K_1 was assumed to have various constant values and the independent variable was $1/(1 + K_1/[H^+])$. Minima were observed at pK_1 7.4 and 7.0 for the conversions of R430 → P380 and P380 → rhodopsin, respectively. The best values for k_{12} and k_{21}, together with their standard errors, are listed in Table I. The variation in K_1 is neglected. The pK values were probably accurate with a deviation of ±0.2.

The pK_1 values 7.4 and 7.0 probably represent a histidine residue of opsin. This value is higher than normal, but the value of pK_a of histidine in the protein varies from 5.5 to 8.8 depending on the effects of surrounding residue [23].

TABLE I

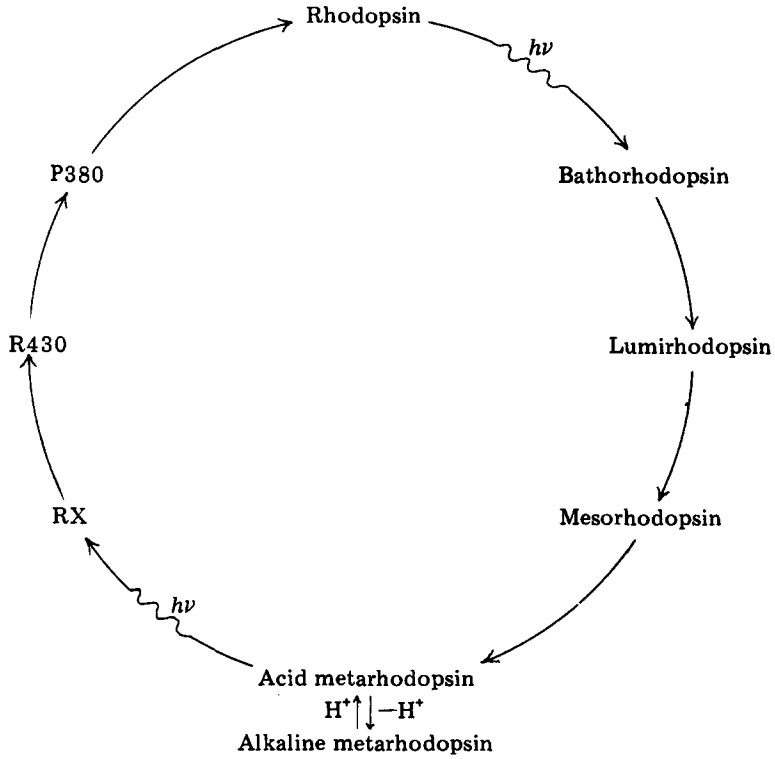
RATE CONSTANTS FOR REGENERATION OF SQUID RHODOPSIN AT 5.5°C IN 0.4% DIGITONIN SOLUTION

Process	k_{12} (s^{-1})	k_{21} (s^{-1})	pKA_1
R430 → P380	27.24 ± 3.71	15.09 ± 0.59	7.4
P380 → rhodopsin	13.92 ± 3.64	1.14 ± 0.50	7.0

Discussion

Kito et al. [8] have found P380 as an intermediate in the photoregeneration process of rhodopsin, when a rhodopsin solution containing $B_4O_7^{2-}$ was irradiated with continuous light longer than 520 nm. The present results show that P380 was also formed upon irradiation with a pulse flash light of a metarhodopsin solution without $B_4O_7^{2-}$. Furthermore, we found an intermediate R430 which is a precursor of P380.

From these results, the squid rhodopsin cycle can be illustrated as follows



The bleaching process of squid rhodopsin involves photochemical and thermal reactions as indicated by low temperature spectroscopy [24—26], high pressure studies [27] and flash photolysis experiments [28,29]. The photoregeneration process also involves both photochemical and thermal reactions, the direction of which must be the reverse of those in the bleaching process. R430, with 11-*cis* retinal as the chromophore, is produced from metarhodopsin in light and is converted to rhodopsin in the dark. Thus, R430 and P380 must be important intermediates in the photoregeneration process of squid rhodopsin.

Transition of metarhodopsin to R430

Metarhodopsin contains all-*trans* retinal as the chromophore attached to opsin. The maximum absorbance peak of squid metarhodopsin shifts with the pH. This is at 490 nm for acid metarhodopsin and 380 nm for alkaline meta-

rhodopsin (the pK of the acid metarhodopsin ⇌ alkaline metarhodopsin equilibrium is 9.1 [30]). In the present experiment, irradiation was carried out with light longer than 540 nm, which alkaline metarhodopsin fails to absorb. Therefore, R430 must be produced from acid metarhodopsin. The chromophore of R430 may be in the 11-*cis* configuration because R430 is produced from acid metarhodopsin by light and regenerates rhodopsin in the dark. R430 appears to be the first detectable photoproduct upon flash illumination of rhodopsin. However, the discontinuity of the kinetic curve in the early stage suggests that the primary photochemical process may be complete within the duration of the flash. This is also suggested by the results of laser photolysis which showed that the life time of photoisomerization of the retinal in cattle rhodopsin was less than 6 ps at a physiological temperature [30].

Transition of R430 to P380

The transient spectrum shown in Curve 2 in Fig. 2 suggests that the Schiff base link in R430 is protonated. On the other hand, the Schiff base link in P380 is considered to be deprotonated. Therefore, a deprotonation step must occur during the conversion of R430 to P380. However, the results shown in Table I suggest that this conversion requires a proton. Eqn. 1 shows that the protolytic reaction is very fast compared with the subsequent isomerization. It is not certain whether the process in question involves an inter- or intramolecular proton transfer. The pK_a value between RH and R^-H^+ was obtained as 7.4 and the ionized group was thought to be a histidine residue of opsin. The slower reaction was considered as the equilibrium between two isomers, R_1H and R_2H, with a concomitant net uptake of protons. The isomerization might consist of the making and breaking of a hydrogen bond between a histidine residue of opsin and some other group of a net positive cluster of basic and acidic groups. This isomerization reaction may induce the configurational rearrangement of the residues around the Schiff base link. This may lead to a disruption of the interaction between the proton-donating residue in opsin and the Schiff base. Such a protonation of opsin, which induces deprotonation of the Schiff base, is also observed during the conversion of Meta I to II of bovine rhodopsin [32].

The transition of R430 to P380 is the first thermal step in the photoregeneration process. The temperature effect on the rates show that the enthalpy and entropy of activation of the conversion are 35.6 kcal/mol and +74.0 cal/deg per mol, respectively. The entropy of activation of this process was three times larger than that of the conversion of P380 → rhodopsin. Apparently, the large positive entropy of activation drives the reaction over the large energy of activation barrier.

Suzuki et al. [10] suggested that the major conformational change of opsin takes place in the transformation from metarhodopsin to P380. Present results show that the major conformational change in opsin in the photoregeneration process takes place in the conversion of R430 to P380.

Transition of P380 to rhodopsin

The pH dependence of the rate of the conversion of P380 to rhodopsin suggests that P380 requires a proton in its conversion to rhodopsin. In this con-

version, the absorption maximum shifts approx. 100 nm to a longer wavelength. This is probably due to the protonation of the Schiff base link between retinal and opsin. However, this reaction can proceed even at pH 10.1, where there is hardly any protonated Schiff base in free solution. Due to the fact that photoregeneration via P380 is not affected by NH_2OH and $NaBH_4$ [9], the Schiff base link in P380 has been thought to be embedded in opsin as it is in rhodopsin. The findings suggest that the proton donor is one of the residues around the Schiff base link in opsin and not a free proton in solution. Kinetic data suggest that the conversion of P380 to rhodopsin was accompanied by the net uptake of a proton by a group with a pK_a of 7.0 (possibly an imidazole group of a histidine residue of opsin). A protein conformational change might take place in the process of R_2H and R_1H in Eqn. 1. In this transformation the proton donor in the vicinity of the Schiff base comes into position to donate a proton to the Schiff base and finally rhodopsin is regenerated. This conformational change in protein is supported by the kinetic parameters shown in Table I. The enthalpy and entropy of activation were about 26.8 kcal/mol and 36.9 cal/deg per mol. This reaction, therefore, presumably involves yet another major conformational rearrangement of opsin.

Acknowledgement

The author expresses sincere thanks to Professor Y. Terayama for his encouragement during the course of this work. The author thanks also M. Umeda and Y. Nakaya for their technical assistance and Drs. H. Shichi, Y. Kito and T. Suzuki for reading the manuscript and for many helpful discussions. His thanks are due to Professor I. Yamazaki for his permission to use a rapid-scan spectrophotometer. This work was supported in part by grants from the Japanese Ministry of Education.

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