Metaretinochrome in Membranes as an Effective Donor of 11-*cis* Retinal for the Synthesis of Squid Rhodopsin

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ABSTRACT Aporetinochrome, which is a protein moiety of retinochrome without chromophore retinal, is found in the membrane containing retinochrome. All of the prosthetic retinal of retinochrome in membranes, which is all-*trans* retinal, is bound to the chromophoric site on the protein moiety, with protonated Schiff bases showing an absorption band with the maximum at 495 nm. On exposure to light, retinochrome is converted to metaretinochrome at room temperature. The prosthetic retinals of metaretinochrome in membranes, which are 11-*cis* retinals, are in two states: retinals bound to the chromophoric site with protonated Schiff bases, and the free retinals, which are separated from the protein moiety. These states are suggested from the following observations. (*a*) The ratio of the absorbance at 470 nm of metaretinochrome to that at 495 nm of the parental retinochrome differs because of differences in samples and is higher in the purer preparations. (*b*) The difference spectrum of absorption of metaretinochrome caused by alkalinization shows two minimum peaks at ~420 and 470 nm. (*c*) The rate of bleaching of metaretinochrome in membranes with dilute NH_2OH is much faster than that of retinochrome, and the absorption band in the near-UV region is more susceptible to NH_2OH than the visible absorption band. The state of the prosthetic retinals in metaretinochrome was confirmed directly by the reaction of metaretinochrome in membranes with $NaBH_4$. After treatment with $NaBH_4$, the sodium dodecyl sulfate-polyacrylamide gel electrophoretic pattern shows two fluorescent bands: one at the position that corresponds to the retinochrome protein (mol wt 27,000 ± 2,000), and another at the front of migration, where no band of protein is observed. Retinoids extracted from the $NaBH_4$-treated metaretinochrome in membranes and analyzed with high-pressure liquid chromatography show a main peak of 11-*cis* retinol. The results of this and earlier (Seki et al., 1982) papers are summarized, and it is strongly suggested that metaretinochrome in the squid retina may play the role of 11-*cis* retinal donor for opsin and contribute to the synthesis of the squid rhodopsin.

INTRODUCTION

Animal visual pigment is a membrane protein containing 11-*cis* retinal or 11-*cis* 3-dehydroretinal as the chromophore. Light-induced geometric isomerization of

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the chromophore from 11-*cis* to all-*trans* is a trigger of the visual process. In spite of such an important role of the 11-*cis* isomer in the photoreception, the mechanism by which it is supplied to the visual pigment is not clear. In regard to this problem, retinochrome found in the visual cells of cephalopods by Hara and Hara (1965) and Hara et al. (1967) may be significant. Retinochrome is a membrane protein containing all-*trans* retinal as the chromophore and the prosthetic retinal is isomerized to 11-*cis* by light (Hara et al., 1967). The reverse photoisomerization of the chromophore in visual pigments and retinochrome has stimulated interest in the physiological functions of retinochrome in reference to rhodopsin (Hara and Hara, 1968, 1972; Sperling and Hubbard, 1975; Hamdorf, 1979). Nevertheless, since the prosthetic retinal of the final photoproduct of the cephalopod rhodopsin, metarhodopsin, was stably bound to the protein moiety at physiological temperature (Hubbard and St. George, 1958), metarhodopsin did not react with the photoproduct of retinochrome, and the regenerable protein part of the cephalopod rhodopsin, opsin, could not be produced. The relationship between rhodopsin and retinochrome, therefore, has only been speculated upon.

In an earlier paper (Seki et al., 1980*a*), it was reported that regenerable squid opsin could be produced by using a membrane preparation of rhodopsin as the starting material and irradiating it with an intense orange light in the presence of 200 mM NH_2OH. Reconstitution of the squid rhodopsin from the prepared opsin was successful not only with the 11-*cis* retinal formed by photoisomerization in ethanol but also with the 11-*cis* retinal formed by the photoisomerase activity of retinochrome in membranes (Seki et al., 1980*b*). In a previous paper (Seki et al., 1982), it was shown that the photoproduct of retinochrome in membranes provides 11-*cis* retinal for opsin in membranes during the close contact of the membranes without fusion, which results in the reconstitution of squid rhodopsin. The photoproduct of retinochrome at room temperature has been called metaretinochrome (Hara et al., 1981; Seki et al., 1982). Metaretinochrome in membranes was considered from the above results to contribute to rhodopsin synthesis as an effective donor of 11-*cis* retinal to opsin, but why metaretinochrome provides opsin so efficiently with the 11-*cis* retinal remains to be clarified.

In this paper, I will compare some properties of retinochrome and metaretinochrome in membranes and examine the existing state of the prosthetic retinals in metaretinochrome in membranes. It will be shown that some of the prosthetic retinals of metaretinochrome are separated from the protein moiety as free retinals. The results of this and earlier papers are summarized, and it is strongly suggested that metaretinochrome in membranes contributes to the synthesis of squid rhodopsin in the visual cells.

Preliminary accounts on this subject have been presented elsewhere (Seki et al., 1981) and discussed at the Fifth International Congress of Eye Research held in The Netherlands in October, 1982.

MATERIALS AND METHODS

Preparation of Retinochrome in Membranes

The excised eyes or the cephalic parts containing eyes of squid, *Todarodes pacificus*, caught

in the Sea of Japan in June, were stored in a freezer until use. The anterior halves of the eyes were removed and the resulting eyecups were stirred vigorously in a buffered saline (5% [wt/vol] NaCl in 67 mM Na/K phosphate buffer at pH 6.5). Outer segments of the visual cells were dispersed in the buffered saline and the outer segment-free retinas remained in the eyecups. Retinochrome-containing membranes (abbreviated to retinochrome membranes) were prepared from the outer segment-free retinas by the routine method (Seki et al., 1982). A homogenate of the outer segment-free retinas in a 10% sucrose solution in the buffered saline (NaCl/10% sucrose) was centrifuged for 5 min at 3,000 rpm and the supernate was recentrifuged at 12,000 rpm for 20 min. The precipitate was suspended in NaCl/40% sucrose solution and centrifuged at 12,000 rpm for 20 min to float the retinochrome membranes. After two flotations, the membranes in the supernate were collected by twofold dilution with the buffered saline and centrifugation at 12,000 rpm for 1 h. The precipitate was resuspended in NaCl/40% sucrose solution and centrifuged in a stepwise sucrose density gradient with a swinging rotor (RPS 27-2; Hitachi, Tokyo, Japan) at 26,000 rpm for 10 h. The membranous fraction containing the most retinochrome at the interface of NaCl/28% and NaCl/35% sucrose was collected, resuspended in NaCl/40% sucrose, and recentrifuged in the other stepwise sucrose density gradient. More purified retinochrome membranes, which have substantially no absorption because of cytochrome and ommin, were collected at the interface of NaCl/28% and NaCl/32% sucrose, yielding ~6 optical density (OD) of retinochrome in a 1-ml suspension from 50 eyes of *Todarodes pacificus*. In the course of this procedure, various preparations of retinochrome membranes at different purities were obtained; none of them were contaminated with membranes containing rhodopsin (rhodopsin membranes).

Retinochrome membranes could also be prepared from the outer segments of the visual cells (cf. Hara and Hara, 1976) by the repetitive sucrose flotations in NaCl/40% sucrose and stepwise sucrose density gradient centrifugations (cf. Kito et al., 1982). As rhodopsin membranes are less dense than retinochrome membranes, the membranes at the lower interface in a stepwise sucrose density gradient have a higher ratio of retinochrome to rhodopsin. The membrane fraction gathered at the interface of NaCl/30% and NaCl/40% sucrose was recentrifuged in the second stepwise sucrose density gradient, and the membranes collected at the interface of NaCl/29% and NaCl/35% sucrose were allowed to obtain retinochrome membranes that were less contaminated with rhodopsin membranes. For greater purification, retinochrome membranes were gathered at the interface of NaCl/28% and NaCl/32% sucrose by the third centrifugation. A preparation of retinochrome membranes contaminated with rhodopsin, which amounted to ~10% of retinochrome by absorbance, was obtained, yielding ~8 OD of retinochrome in a 1-ml suspension from 50 eyes of *Todarodes pacificus*.

Spectrophotometry

Absorption spectra of the membrane preparations were measured with a Hitachi 323 recording spectrophotometer by the opal glass method (Seki et al., 1980*b*). After recording the absorption spectrum of retinochrome membranes suspended in 38% sucrose at pH 6.5, light from a 100-W projection lamp through a cutoff filter (>560 nm; VO-56; Toshiba, Tokyo, Japan) was illuminated for 5 min, the absorption spectrum of the photoproduct was measured, and a 1/100 vol of 2 M NH_2OH (freshly neutralized) was added to bleach the pigment completely at room temperature. The difference absorbances caused by the addition of NH_2OH at the absorption maxima of retinochrome (495 nm) and metaretinochrome (470 nm) were measured and the ratio A_{470}/A_{495} was determined.

Aporetinochrome, which is the protein moiety of retinochrome containing no chromophore retinal, was quantified by the following method (Seki et al., 1982). After measuring the absorption spectrum of a suspension of retinochrome membranes in 38%

sucrose, a trace amount of all-*trans* retinal concentrated in ethanol was added at a final concentration of ~1–2 μM to convert aporetinochrome into retinochrome. After incubation for 30 min, the suspension was added to a 1/400 vol of 2 mM NH_2OH and incubated for another 30 min to convert free retinals and random retinylidene Schiff bases into retinal oximes, keeping the retinochrome stable. An increase of the absorbance at 495 nm caused by the addition of all-*trans* retinal and dilute NH_2OH was attributed to retinochrome reconstituted from aporetinochrome. Subsequent addition of a 1/100 vol of 2 M NH_2OH bleached the total retinochrome, which is the sum of aporetinochrome and holoretinochrome initially present in the membrane preparation. The amount of retinochrome was calculated from the difference absorbance at 495 nm using the value of 60,000/M·cm for the molar extinction coefficient.

Quantification of Phosphate

The amount of phosphate in the preparation of retinochrome membranes was assayed by the method of Bartlett (1959) as a measure of the purity of the sample. Preparations of retinochrome membranes in sucrose solutions were washed by centrifugation with 0.1 M Tris-HCl (pH 7.0) three times to remove sucrose thoroughly. The washed membranes were suspended in 0.1 M Tris-HCl, fixed volumes of the suspensions were assayed for the phosphate, and aliquots were assayed spectrophotometrically to determine the ratio A_{470}/A_{495}. To calculate the molar ratio P/total retinochrome, the ratio of aporetinochrome to holoretinochrome was determined in each sample.

Reaction with $NaBH_4$

An aliquot of a preparation of retinochrome membranes in 38% sucrose solution at pH 6.5 was kept in darkness, added to a trace amount of $NaBH_4$ grains in the dark, exposed to orange light (>560 nm) for 5 min, or added to $NaBH_4$ after exposure to the orange light. Each aliquot was kept in darkness after the addition of $NaBH_4$ until the bubbling of hydrogen ceased. The samples were measured for the absorption spectra and applied to sodium dodecyl sulfate-polyacrylamide gels, or the retinoids in each sample were extracted for analysis by high-pressure liquid chromatography (HPLC).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Membrane preparations of retinochrome after treatment as mentioned above were washed with water by centrifugation to remove KH_2PO_4 and $NaBH_4$. The precipitated membranes were added with 4% SDS in 0.1 M sodium phosphate buffer at pH 7.3 and dissolved by sonication for 10 min. The samples were applied to a 7.5% slab gel and electrophoresed according to the method of Weber and Osborn (1969). Immediately after electrophoresis, the fluorescent bands were observed under two black lamps (National FL 20s·BL-B; Matsushita Electric Industrial Co., Ltd., Osaka, Japan) and the gel was stained with Coomassie Brilliant Blue.

HPLC

Identification of retinoids and geometric isomers was carried out by HPLC on a Hitachi 635 chromatograph equipped with a 10- × 250-mm column of Lichrosorb SI-60 (5 μm; E. Merck AG, Darmstadt, Federal Republic of Germany). Absorbance at 320 or 360 nm was monitored with a Hitachi 100-50 spectrophotometer. As an eluent, 30% ethyl ether/0.33% ethanol in hexane was used to separate the peaks of 11-*cis* and 13-*cis* retinol. The flow rate was 6 ml/min (50 kg/cm^2). Retinoids in the membrane preparations were

extracted and analyzed by the retinal oxime method (Groenendijk et al., 1979, 1980; Suzuki and Makino, 1981). Addition of 20 mM NH_2OH to the suspension of retinochrome or metaretinochrome membranes in sucrose solution converts the prosthetic retinals into retinal oximes. The retinoids extracted with dichloromethane and excess hexane were concentrated in the eluent and applied to the HPLC system.

All the procedures described above were carried out under dim red light unless otherwise specified.

RESULTS

Aporetinochrome in Retinochrome Membranes

Aporetinochrome in retinochrome membranes was assayed as described in Materials and Methods and found in all the preparations examined so far. The ratio of aporetinochrome to total retinochrome in the membrane preparation was altered from 0.12 to 0.38 in the different preparations; the mean was 0.26 ± 0.08 (n = 12). The retinochrome membranes prepared from the outer segment-free retinas contained more aporetinochrome than those prepared from the outer segment fraction, which had a mean ratio of 0.17 ± 0.05 (n = 4).

Absorption Spectra of Metaretinochrome in Membranes

We have called the photoproduct of retinochrome at room temperature metaretinochrome (Hara et al., 1981; Seki et al., 1982). On exposure to orange light at room temperature, the visible absorption of the suspension of retinochrome membranes decreases with an increase of absorption in the near-UV region (cf. Figs. 2 and 3). The profile of the absorption spectrum of metaretinochrome has been observed to be different from sample to sample. For a characterization of the absorption spectrum of metaretinochrome, the ratio of the absorbance of metaretinochrome at 470 nm to that of the parental retinochrome at 495 nm (A_{470}/A_{495}) was calculated; the varied values in the range of 0.23 and 0.49, with a mean of 0.37 ± 0.06 (n = 25), were obtained. The correspondence of the values to the purity of the samples was then examined as shown in Fig. 1. As a measure of the purity of retinochrome membranes, the molar ratio of the amount of phosphate to that of total retinochrome in the sample (P/Total Ret) was used. The result clearly indicates that the purer the membrane preparations, the higher the value of A_{470}/A_{495}.

Effect of Alkaline pH on the Absorption Spectra of Retinochrome and Metaretinochrome in Membranes

The spectral change of absorption at alkaline pH was examined with membrane preparations of retinochrome and metaretinochrome. Alkalinization of the suspension of retinochrome membranes in 40% sucrose up to pH 9.9 did not significantly change the profiles of the apparent absorption spectra. In spite of the decrease of the apparent absorption caused by the decrease in the turbidity of the suspension, the difference spectrum was nearly a straight line. As the pH of the suspension was raised further from 10, the profile of the apparent absorption began to change and the difference spectra showed a decrease of

absorption in the visible range with a peak at ~495 nm and an increase in the near-UV with a peak at ~380 nm. The visible absorption band was bleached completely at a pH of >12.

Metaretinochrome in membranes was more susceptible to alkaline pH and showed behavior different from that of retinochrome (Fig. 2). On raising the pH from 6.5 (curve 1) to 8.2 (curve 3), the profile of the apparent absorption changed, and the difference spectrum (curve 3 − 1) showed a decrease of absorption with the minimum at ~420 nm. Subsequently raising the pH to 10.6 (curve 6) also decreased the visible absorption, but the difference spectrum reached a minimum at ~470 nm (curve 9 − 3). Raising the pH further from

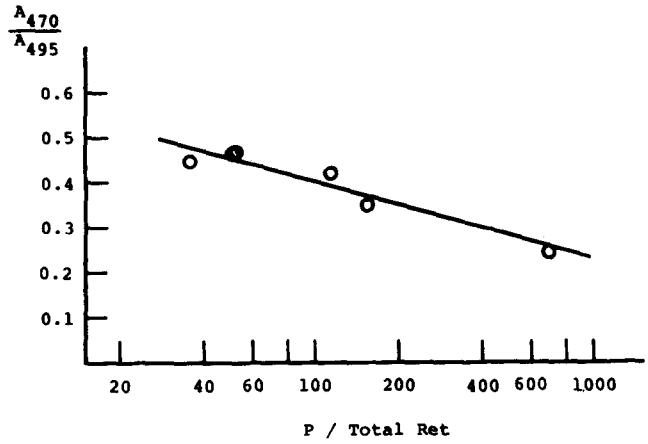

FIGURE 1. Relationship between the purity of the preparation of retinochrome membranes and the absorption spectrum of metaretinochrome. After washing with 0.1 M Tris-HCl buffer at pH 7.0, the samples of retinochrome membranes were assayed for the amount of phosphate and the ratio A_{470}/A_{495} (see Materials and Methods). The molar ratio of phosphate to total retinochrome (P/Total Ret) was determined, and A_{470}/A_{495} was plotted as a function of log(P/Total Ret). The purer the preparation of retinochrome membranes was, the higher the visible absorption of the photoproduct was.

10.6 decreased the apparent absorption at all wavelengths almost equally; the difference spectrum (curve 9 − 6) was nearly a straight line. These results suggest that in metaretinochrome the prosthetic retinals are in at least two states.

Reaction of Retinochrome and Metaretinochrome in Membranes with NH_2OH at Low Concentrations

Both retinochrome and metaretinochrome are easily and completely bleached by addition of 20 mM NH_2OH, and retinal oximes of the corresponding retinal isomers are produced (cf. Fig. 5, *A* and *B*). The bleaching reaction strongly depends on the concentration of NH_2OH added. When an aliquot of a suspension of retinochrome membranes in 40% sucrose (2.4 μM retinochrome) was added to 1.3 mM NH_2OH, the visible absorption of retinochrome decreased gradually,

with an isosbestic point at 410 nm, taking ~40 min to reach completion. When further-diluted NH_2OH (33.3 μM) was added, retinochrome bleached only ~20% after 90 min and 50% overnight. Metaretinochrome in membranes, on the other hand, bleached substantially in the presence of 33.3 μM NH_2OH in 90 min (Fig. 3). The bleaching process in metaretinochrome was not as simple as that in retinochrome. Immediately after addition of NH_2OH, absorption at ~410 nm decreased, with the isosbestic point at ~370 nm (curve 2), and thereafter visible absorption bleached with an isosbestic point at ~395 nm (from curve 2 to curve

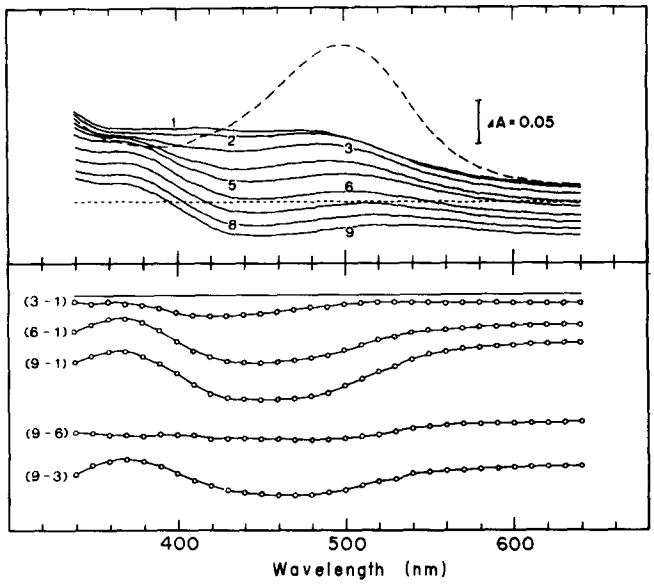

FIGURE 2. Change in the absorption spectrum of metaretinochrome in membranes at alkaline pH's. A suspension of retinochrome membranes in 40% sucrose at pH 6.5 (broken line) was exposed to orange light exceeding 560 nm for 5 min (curve 1) and the absorption spectrum was recorded at pH 6.90 (2), 8.15 (3), 9.60 (4), 10.12 (5), 10.61 (6), 10.97 (7), 11.31 (8), or 11.60 (9) successively. The difference spectra between curves 1 and 3 (3 − 1), 1 and 6 (6 − 1), 1 and 9 (9 − 1), 6 and 9 (9 − 6), and 3 and 9 (9 − 3) are shown in the lower column. On raising the pH of the suspension, the turbidity decreased; the apparent absorption at ~640 nm decreased and exceeded the baseline (dotted line) at pH's of >10.

9). The time courses of bleaching retinochrome with NH_2OH plotted under pseudo–first-order kinetics followed straight lines with k_1 at 2.37×10^{-3} min^{-1} (33.3 μM NH_2OH), 1.75×10^{-2} min^{-1} (133 μM NH_2OH), and 9.85×10^{-2} min^{-1} (1.3 mM NH_2OH). The kinetics of bleaching metaretinochrome in the early phases (from 1 to 10 min) followed straight lines with k_1 at 2.37×10^{-2} min^{-1} (133 μM NH_2OH), 5.21×10^{-2} min^{-1} (33.3 μM NH_2OH), and 14.5×10^{-2} min^{-1} (133 μM NH_2OH); metaretinochrome bleached almost 20 times as fast as retinochrome. It is obvious from the results that the prosthetic retinals of metaretinochrome are more accessible to the hydrophilic molecules in the solvent

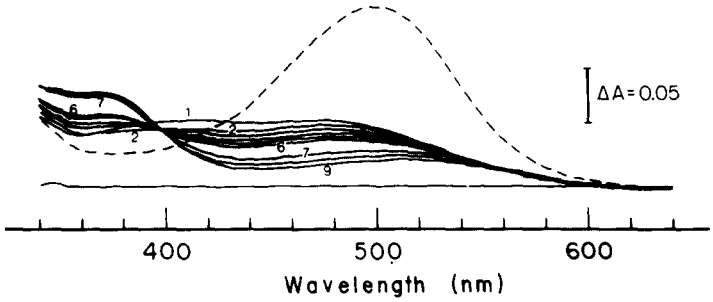

FIGURE 3. Bleaching of metaretinochrome in membranes with NH_2OH at low concentration. A suspension of retinochrome membranes in 1.5 ml of 40% sucrose at pH 6.5 (broken line; 2.4 μM retinochrome) was exposed to orange light exceeding 560 nm (curve 1) and added to 5 μl of 10 mM NH_2OH. The absorption spectrum was measured repeatedly at 1 min (curve 2), at successive intervals of 2 min, 20 s (from curve 3 to curve 6), at 30 min (7), at 90 min (8), and overnight (9) after addition of NH_2OH.

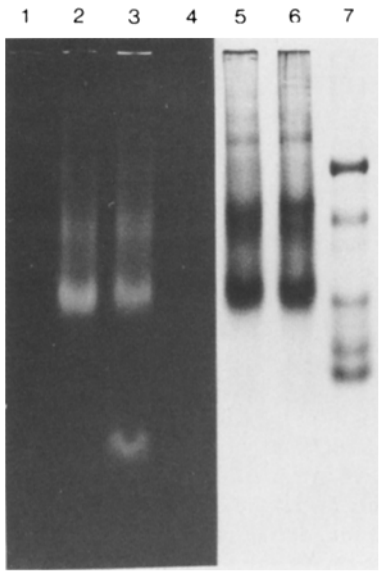

FIGURE 4. SDS-PAGE patterns of retinochrome and metaretinochrome membranes before and after treatment with $NaBH_4$. Fluorescent bands (left column) were observed under two black lamps immediately after electrophoresis of retinochrome membranes before (1) or after (2) addition of $NaBH_4$, and metaretinochrome membranes before (4) or after (3) addition of $NaBH_4$. Protein bands (right column) were stained with Coomassie Brilliant Blue. Lanes 5 and 6 are same as lanes 3 and 4, respectively. Lane 7 shows the bands of marker proteins (top to bottom): bovine serum albumin (mol wt 66,000), ovalbumin (45,000), trypsinogen (24,000), β-lactoglobulin (18,400), and lysozyme (14,300).

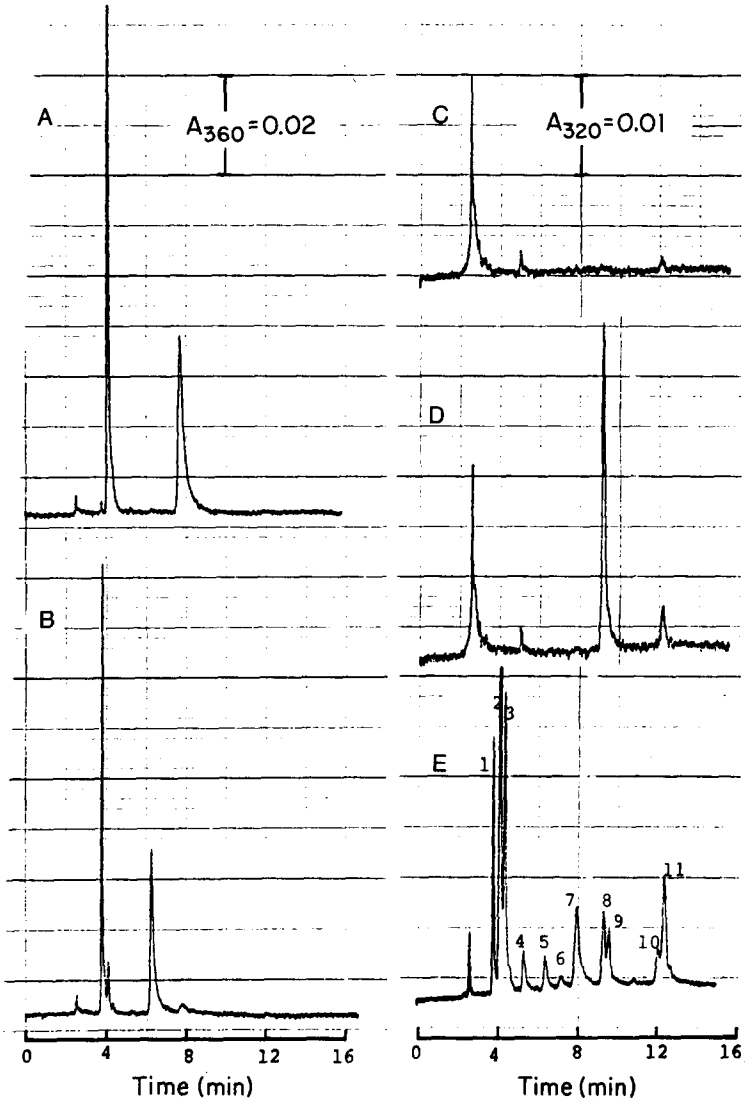

FIGURE 5. High-pressure liquid chromatograms of the extracts from retinochrome membranes (*A*), metaretinochrome membranes (*B*), retinochrome membranes treated with $NaBH_4$ (*C*), and metaretinochrome membranes treated with $NaBH_4$ (*D*). The samples were added to 20 mM NH_2OH, extracted by CH_2Cl_2 and hexane, and applied to the column of Lichrosorb SI-60 (10 × 250 mm). Elution was performed at a flow rate of 6 ml/min with 30% ethyl ether/0.33% ethanol in hexane (vol/vol). The peaks were detected at 360 nm for *A*, *B*, and *E* and at 320 nm for *C* and *D*. Peaks of standard oximes and retinols (*E*) are: (1) syn 11-*cis*; (2) syn all-*trans*; (3) syn 13-*cis* plus 9-*cis*; (4) anti 13-*cis*; (5) anti 11-*cis*; (6) anti 9-*cis*; (7) anti all-*trans* retinal oximes; (8) 11-*cis*; (9) 13-*cis*; (10) 9-*cis*; (11) all-*trans* retinols.

than those of retinochrome, and, in contrast to those of retinochrome, they are not in a single state.

Reaction of Retinochrome and Metaretinochrome in Membranes with $NaBH_4$

For examination of the state of the prosthetic retinals in retinochrome and metaretinochrome, $NaBH_4$ is a useful reagent. Retinals forming Schiff base linkages with amino groups are reduced to *N*-retinyl products (Bownds and Wald, 1965) and free retinals are reduced to retinols; both the products fluoresce under near-UV light. As Hara and Hara (1973) have shown with solubilized samples, both retinochrome and metaretinochrome in membranes react easily with $NaBH_4$ at neutral pH, forming the products with maxima at ~350 and 340 nm, respectively. In order to identify the products, samples solubilized in SDS were applied to a slab gel for SDS-PAGE and observed under black lamps immediately after electrophoresis (left column in Fig. 4). Without treatment with $NaBH_4$, neither retinochrome nor metaretinochrome showed fluorescent bands (lanes 1 and 4). Lane 2 shows that $NaBH_4$-treated retinochrome has a remarkable fluorescent band at the position corresponding to the band of retinochrome protein shown in the lanes 5 and 6 (mol wt 27,000 ± 2,000). All the prosthetic retinal of retinochrome must have attached to the protein moiety with Schiff base and reduced to *N*-retinyl protein. Lane 3 shows the electrophoretic pattern of $NaBH_4$-treated metaretinochrome. A fluorescent band other than the *N*-retinyl protein band is clearly observed near the front of migration, where no protein band is observed. A part of the prosthetic retinals of metaretinochrome must have separated from the protein moiety and another part must have formed Schiff bases with the protein moiety.

Retinoids in retinochrome or metaretinochrome membranes before and after addition of $NaBH_4$ were extracted and analyzed with HPLC by the oxime method (Fig. 5). Fig. 5, *A* and *B*, shows that before addition of $NaBH_4$, substantially all (98%) of the prosthetic retinal of retinochrome is all-*trans* and 97% of the retinal is isomerized to 11-*cis* by irradiation with orange light exceeding 560 nm. After addition of $NaBH_4$, the extract from retinochrome membranes had no peaks of retinal oxime or retinol (Fig. 5*C*), but the extract from metaretinochrome membranes (Fig. 5*D*) showed a main peak of 11-*cis* retinol with a little peak of all-*trans* retinol. The results indicate clearly that in metaretinochrome, some of the prosthetic retinals are present as free 11-*cis* retinals and are reduced to retinol by $NaBH_4$.

DISCUSSION

Retinochrome in membranes of *Todarodes pacificus* has an absorption maximum at 495 nm (Figs. 2 and 3) and has all-*trans* retinal as the prosthetic retinal (Fig. 5*A*). The results shown in Figs. 4 (lane 2) and 5*C* indicate that all of the prosthetic retinal is bound to the protein moiety. The chromophore of retinochrome has been proposed to be a protonated Schiff base of all-*trans* retinal with an amino group of the protein moiety (Hara and Hara, 1973; Azuma et al., 1975; Hubbard and Sperling, 1973; Sperling and Hubbard, 1975). As the acid-denatured retino-

chrome has an absorption maximum at 445–450 nm (Hara and Hara, 1973; Azuma et al., 1974), the prosthetic retinal of retinochrome must have a secondary interaction with the protein moiety (cf. Hubbard and Sperling, 1973). In comparison with the chromophore of metarhodopsin in membranes, which bleaches gradually with 0.2 M NH_2OH and takes ~2 h to bleach completely (T. Seki, unpublished observation), the chromophore of retinochrome in membranes, which bleaches easily with 20 mM NH_2OH, is more accessible to the small hydrophilic molecules in the solvent. On the other hand, when the absorption spectra of retinochrome and metarhodopsin in each membrane were compared at pH 11.6, metarhodopsin had bleached completely (cf. Seki et al., 1980*b*), but retinochrome had not. These results indicate that the chromophore of retinochrome is not directly affected by the alkaline pH, but is affected indirectly, probably through the conformational change of the protein structure.

On exposure to orange light exceeding 560 nm at room temperature, retinochrome is converted to metaretinochrome. The absorption spectrum of metaretinochrome has an absorption maximum at ~470 nm and a secondary absorption band in the near-UV region; the profile of the absorption spectrum at neutral pH differs from sample to sample. The geometric configuration of 97% of the chromophore retinals of metaretinochrome after exposure to orange light is 11-*cis* retinal (Fig. 5*B*). The characteristic profile of the absorption spectrum of metaretinochrome is therefore not attributed to the mixture of the geometric configurations of the retinals but must be attributed to the state of the prosthetic retinals. At least two states of the prosthetic retinals in metaretinochrome were suggested by their reactivity with alkaline pH (Fig. 2) and NH_2OH at low concentrations (Fig. 3). The absorption band at ~470 nm, which is optically active (Azuma et al., 1974), is considered to be due to 11-*cis* retinal bound to the chromophoric site on the protein moiety with protonated Schiff base. The retinals bound to the protein moieties with Schiff bases are reduced to *N*-retinyl proteins, as shown in Fig. 4, lane 3. The absorption band of metaretinochrome in the near-UV region was affected more easily by alkaline pH (Fig. 2) or by dilute NH_2OH (Fig. 3) than the visible absorption band. Azuma et al. (1974) have suggested from the results of circular dichroism and the difference absorption spectrum that some of the 11-*cis* retinals of metaretinochrome might have been released from the binding site of the protein part as free retinals. The results shown in Fig. 5 are evidence that some (20–40% because of samples) of the prosthetic retinals in metaretinochrome are present as free 11-*cis* retinals and are reduced to retinols by $NaBH_4$.

Photoregeneration of retinochrome from metaretinochrome by exposure to near-UV or blue light (Hara and Hara, 1972) may be explained by such free 11-*cis* retinals as are isomerized by the light to all-*trans* retinals.

In Fig. 1, the ratio A_{470}/A_{495} was used to characterize the absorption spectrum of metaretinochrome. The ratio is interpreted as a function of the ratio of the bound retinals in metaretinochrome to all the prosthetic retinals of the parental retinochrome. On the basis of this concept, the result shown in Fig. 1 is explained as follows: a greater fraction of the prosthetic retinals of metaretinochrome is

released from the protein moieties in a heavily contaminated preparation than in a purer preparation. It is probably due to the migration of the free retinals in metaretinochrome membranes to the other membranes. In an earlier paper (Seki et al., 1982), it was shown that as much as 80% of the prosthetic retinals of metaretinochrome are used for the reconstitution of rhodopsin in the mixture of opsin membranes and metaretinochrome membranes. These results indicate that removal of the free retinals from the metaretinochrome membranes decreases the amount of the bound retinals that show the absorption band with a maximum at 470 nm. This state of prosthetic retinals in metaretinochrome may also explain the mechanism of "spontaneous regeneration" of retinochrome from metaretinochrome. In the dark, metaretinochrome reverts slowly to retinochrome; up to 70–90% of retinochrome initially present is regenerated in 1–3 d at 25–30°C (Sperling and Hubbard, 1975; Hara et al., 1981; Ozaki et al., 1983). A fraction of the free retinals in metaretinochrome would be isomerized thermally from 11-*cis* to all-*trans* retinals and would be combined with aporetinochrome, which would result in the slow accumulation of retinochrome.

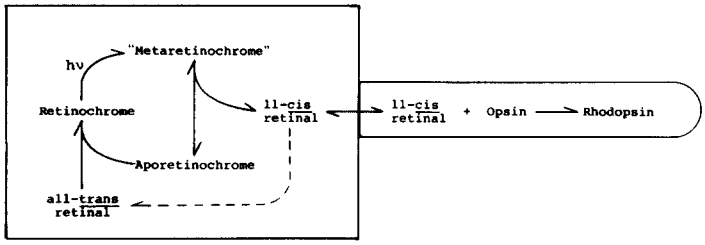

FIGURE 6. Schematic representation of the relationship between metaretinochrome in a membrane and opsin in the other membrane. "Metaretinochrome" indicates the state of 11-*cis* retinal bound to the protein moiety (aporetinochrome) showing an absorption maximum at 470 nm. See text for more details.

The results obtained so far on the retinochrome in membranes are summarized in Fig. 6. Retinochrome is a complex of all-*trans* retinal and aporetinochrome, with the absorption maximum at 495 nm at neutral pH. On exposure to orange light exceeding 560 nm, retinochrome is converted to metaretinochrome at room temperature. In metaretinochrome, the isomeric configuration of the prosthetic retinal is 11-*cis* and the retinal is in two states: the free retinal and the retinal bound to the protein moiety. The state of 11-*cis* retinal bound to the chromophoric site, probably with protonated Schiff base, which shows the absorption maximum at 470 nm, has been described as "Metaretinochrome" in Fig. 6. In the presence of opsin membrane in the reaction mixture, the free 11-*cis* retinal in the metaretinochrome membrane migrates to the opsin membrane during the direct contact of the membranes. The combination of 11-*cis* retinal with opsin results in the formation of rhodopsin. Part of the aporetinochrome found in the retinochrome membranes may be the product of metaretinochrome, which provided the prosthetic retinal for opsin in the squid retina. The broken

line in the figure shows the thermal or photic isomerization of free 11-*cis* retinal to all-*trans* retinal. Recombination of all-*trans* retinal with aporetinochrome results in the regeneration of retinochrome.

This scheme strongly suggests the possibility that metaretinochrome in the squid retina may play the role of 11-*cis* retinal donor for the squid opsin and may contribute to the rhodopsin synthesis.

The feasibility of this idea will be tested by quantification of retinochrome, metaretinochrome, rhodopsin, and metarhodopsin in the squid retina under various light conditions by the oxime method using HPLC.

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