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Studies on Structure and Function of Rhodopsin by Use of Cyclopentatrienylidene 11-*cis*-Locked-rhodopsin†

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ABSTRACT: The photochemical reaction of cyclopentatrienylidene 11-*cis*-locked-rhodopsin derived from cyclopentatrienylidene 11-*cis*-locked-retinal and cattle opsin was spectrophotometrically studied. The difference absorption spectrum between the cyclopentatrienylidene 11-*cis*-locked-rhodopsin and its retinal oxime had its maximum at 495 nm (P-495). Irradiation of P-495 at −196 °C with either blue light or orange light caused no spectral change, supporting the cis–trans isomerization hypothesis for formation of bathorhodopsin. Upon irradiation of P-495 at 0 °C with orange light, however, its absorption spectrum shifted to a shorter wavelength owing to formation of a hypsochromic product. The difference absorption spectrum between this product (P-466) and its retinal oxime showed its maximum at 466 nm. Analysis of retinal isomers by high-performance liquid chromatography showed that this spectral shift was not accompanied by photoisomerization of the chromophore. P-466 could almost completely be photoconverted to the original pigment (P-495) by irradiation at 0 °C with blue light with little formation of the other isomeric form of its chromophore. The α-band of the circular dichroism spectrum of P-495 was very small in comparison with that of rhodopsin, while that of P-466 was comparable to it. These facts suggest that P-495 has a planar conformation in the side chain of the chromophore and that P-466 has a twisted one, probably at the C_8–C_9 single bond. Cyclic-GMP phosphodiesterase in frog rod outer segment was activated by neither P-495 nor P-466. This result suggests that the isomerization of the retinylidene chromophore of rhodopsin is indispensable in the phototransduction process.

It has been proposed that a photon absorbed by rhodopsin isomerizes the 11-*cis*-retinylidene chromophore to an all-trans form (Hubbard & Kropf, 1958). Later, one of us (Yoshizawa & Wald, 1963) suggested that an early photoproduct of rhodopsin photolysis, bathorhodopsin (formerly called prelumirhodopsin), might possess a twisted form of *all-trans*-retinal as its chromophore and extended the cis–trans photoisomerization hypothesis. In order to obtain direct evidence for this hypothesis, two kinds of retinal analogues have recently been synthesized in which rotation around the C_{11}–C_{12} double bond is blocked because of a fixed chemical bond: one is locked in the 11-cis conformation with cycloheptene (seven-membered ring; Akita et al., 1980) and the other with cyclopentene (five-membered ring; Ito et al., 1982). Both analogues bind with cattle opsin to produce rhodopsin analogues having their λ_{max}s at 490 (Akita et al., 1980) and 498 nm (Ito et al., 1982), respectively. Low-temperature spectrophotometric study of an artificial pigment derived from the former analogue and cattle opsin (Mao et al., 1981) showed the lack of a bathointermediate. This paper also shows that cyclopentatrienylidene 11-*cis*-locked-rhodopsin[1] (we tentatively call this analogue, having a five-membered ring in the side chain of the chromophore, Rh5) prepared from cyclopentatrienylidene 11-*cis*-locked-retinal (Ret5; Figure 1) and cattle opsin yields no bathointermediate and supports the cis–trans isomerization hypothesis as to the formation of bathorhodopsin.

Another aim of this study using this rhodopsin analogue is to demonstrate the origin of induced circular dichroism (CD) of rhodopsin. 11-*cis*-Retinal in free form has its absorption maximum in the near-ultraviolet spectral region but displays no optical activity. Binding of it to opsin induces optical activity in the visible and near-ultraviolet spectral regions with a large red shift of absorption maximum from 370 to 498 nm (Hubbard et al., 1965; Crescitelli et al., 1966). So far, three different explanations for the origin of the induced CD of rhodopsin have been proposed (Ebrey & Yoshizawa, 1973; Honig et al., 1973; Kropf et al., 1973). One of them attributed it to a twisted 11-*cis*-retinylidene chromophore, because calculations showed that 11-*cis*-retinal has at least two twisted single bonds at C_6–C_7 and at C_{12}–C_{13} due to steric hindrances (Honig et al., 1973) between the H atom of C_8 and the methyl group of C_5 and between the H atom of C_{10} and the methyl group of C_{13}, respectively. Supposedly, one of the conformers of 11-*cis*-retinal may selectively bind to the retinal-binding site of opsin. Thus, the optical activity of rhodopsin may be ex-

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[1] Cyclopentatrienylidene is a term used loosely, rather than rigorously, to indicate the presence of a 3,5-cyclopentenediylidene group. Thus, cyclopentatrienylidene 11-*cis*-locked-retinal is not retinal with a cyclopentatrienylidene substituent but rather is retinal containing this system.

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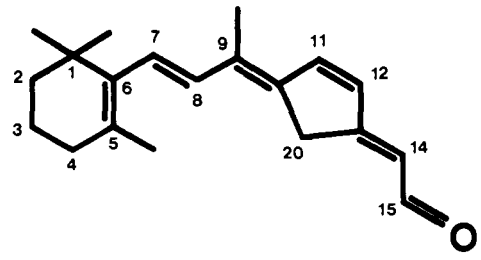

FIGURE 1: Structure of Ret5.

plained by an asymmetric conformation of the retinylidene chromophore derived from such a selective binding. In another explanation, it is ascribed to an asymmetric structure of opsin, to which retinal can bind by changing its conformation (Takezaki & Kito, 1967). Either of these two explanations may be called the "intrinsic model" because the optical activity is attributed to a twisted structure of the retinylidene chromophore in the retinal-binding site of opsin. If this is the case, Rh5 should display no or only a little optical activity, because the chromophore of Rh5, Ret5, has a full planar conformation in the side chain moiety from C_9 to C_{14} (see Figure 1), judging from a ^1H NMR study and a UV maximum value of the absorption spectrum (Ito et al., 1982). The third explanation is that the induced CD is derived from coupling of two oscillators ("coupled oscillator model"; Kagi et al., 1971; Waggoner & Stryer, 1971), that is, a dipole–dipole interaction between the retinylidene chromophore and some amino acids of opsin, presumably one or more of the tryptophyl residues (Kropf et al., 1973). If Ret5 binds to the 11-*cis*-retinal-binding site of opsin, it should act as a dipole that is not so much different from 11-*cis*-retinal in both oscillator strength and azimuthal angle to another oscillator on the opsin moiety. On this basis, it is expected that Rh5 might exhibit a comparable CD spectrum with that of rhodopsin. In order to clarify the origin of the induced CD of rhodopsin, the CD spectrum of Rh5 has been examined.

The last aim of this paper is to show that cis–trans photoisomerization is an essential step in making the physiological activity of rhodopsin manifest. When rhodopsin absorbs a photon, it bleaches over a chain of intermediates, one or more of which triggers a series of biochemical reactions in the intra- and interdisc space of rod outer segment. Finally, the sodium channels of its plasma membrane are presumably shut to stop the sodium current (Sillman et al., 1969). One hypothetical linkage between rhodopsin photolysis in the disc membrane and the ionic permeability change of the plasma membrane has been proposed in which guanosine cyclic 3′,5′-monophosphate (cGMP) plays a role as an intracellular transmitter (Hubbell & Bownds, 1979; Pober & Bitensky, 1979). In this hypothesis, flow of information involves an activation of cGMP phosphodiesterase. Here, we have measured the cGMP phosphodiesterase activation abilities of Rh5 and its derivatives in the presence of both GTP and GTP-binding protein to examine their physiological activities.

Materials and Methods

All manipulations unless otherwise stated were done under ice-chilled conditions.

Preparation of Cattle Opsin. All the procedures before irradiation of the sample were performed under dim red light. Rod outer segments were isolated from dark-adapted cattle retinas by the method of Matsumoto et al. (1978) in 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.0). They were suspended in the HEPES buffer containing 100 mM hydroxylamine (NH_2OH; pH 7.0) and irradiated with orange light from a 2-kW tungsten lamp passing through a Toshiba VO-56 cutoff filter (>540 nm) in order to bleach rhodopsin completely. After being washed 10 times with the HEPES buffer to remove NH_2OH and 2 times with distilled water, the pellet was lyophilized, followed by washing it 5 times with petroleum ether (bp 30–60 °C) to remove retinal oxime and some lipids. The opsin membrane thus obtained was solubilized with 2% "soluble" digitonin (Bridges, 1977) dissolved in the HEPES buffer (pH 7.0) and then spun down to get a clear supernatant. The opsin concentration was estimated by converting it to rhodopsin (ϵ = 40 600 at 498 nm; Wald & Brown, 1953) in the presence of excess 11-*cis*-retinal.

Preparation of Ret5. Ret5 and other two isomers were synthesized from β-ionyl sulfone and 4-acetoxycyclopentenone as previously described (Ito et al., 1982). The geometry of each isomer was characterized by ^1H NMR. The Ret5 thus obtained was dissolved in hexane and stored at −70 °C in an argon atmosphere just before use.

Preparation of Rh5. Ret5 dissolved in hexane was dried up under a N_2 stream and then dissolved in ethanol to give about a 50 mM concentration. [The molar extinction coefficient of Ret5 at 405 nm is 18 500 cm^{-1} M^{-1} in ethanol (Ito et al., 1982).] Molar excesses of Ret5 of 2–5-fold in ethanol (1 volume) were mixed with cattle opsin in 2% digitonin solution (500 volumes) and then incubated for 20 h at 20 °C to generate Rh5. In order to remove excess Ret5, this solution containing Rh5 was applied to a concanavalin A–Sepharose 4B affinity column (1.2 cm in diameter and 5.0 cm long), which had been equilibrated with 10 mM HEPES buffer (pH 7.0) containing 2% digitonin, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (buffer A). After the column bed was well washed with buffer A, Rh5 was eluted with buffer A containing 150 mM methyl α-mannoside. The methyl α-mannoside and other ions in the eluate were removed by means of flow dialysis with an ultrafiltration membrane (YM-30 from Amicon) to get a purified Rh5 in 2% digitonin buffered with 10 mM HEPES (pH 7.0). As a control sample, regenerated rhodopsin (11-*cis*-rhodopsin) was prepared from another aliquot of the same cattle opsin and 11-*cis*-retinal by incubation under the same conditions as were used with Rh5.

Preparation of Schiff-Base Compound of Ret5 with n-Butylamine. Ret5 (1 nmol) dissolved in 20 μL of hexane was dried under a N_2 stream. To the residue obtained was added 50 μL of *n*-butylamine. The reaction tube sealed under a N_2 atmosphere was shaken vigorously and stored at 0 °C for 3 h in the dark. Then, the sample was dried under a N_2 stream and dissolved in 1 mL of methanol. The Schiff base formed between Ret5 and *n*-butylamine was protonated by addition of 2.5 μL of 10 N HCl. In the course of protonation of the Schiff base, the absorption maximum of the sample shifted from 353 to 464 nm.

High-Performance Liquid Chromatographic Analysis of Extracted Chromophore. Extraction of the chromophores of Rh5 and its derivatives was performed by the method of Maeda et al. (1977) under dim red light. The sample (absorbance at 495 nm = 0.2) in 2 mL of 2% digitonin buffered with 10 mM HEPES (pH 7.0) was mixed with an equal volume of CH_2Cl_2. After vigorous trituration for 8 min by use of ultraturrax (Ika-Werke), a 4-fold volume of chilled petroleum ether was added and triturated again for 2 min. Then, the petroleum ether was transferred to another tube, dried over anhydrous Na_2SO_4, and evaporated with a N_2 stream. The residue was dissolved in 100 μL of *n*-heptane and then analyzed by high-performance liquid chromatography

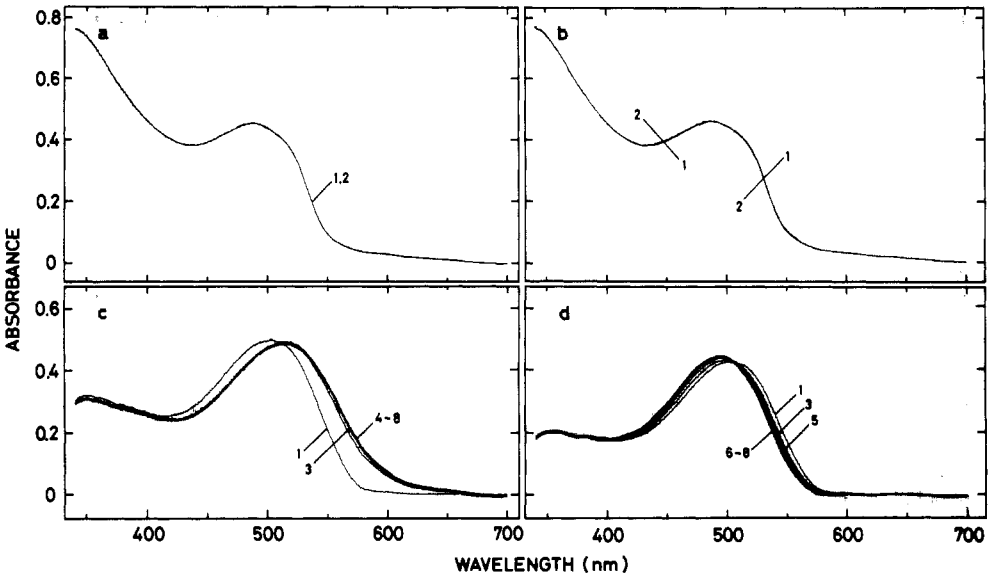

FIGURE 2: Photoreaction of Rh5 and rhodopsin at −196 °C. (a) Rh5 (curve 1) was irradiated with blue light at 433 nm for 64 min (curve 2). (b) Rh5 (curve 1) was irradiated with orange light at wavelengths longer than 560 nm for 64 min (curve 2). (c) Rhodopsin (curve 1) was successively irradiated with the blue light for a total of 1, 2, 4, 8, 16, 32, and 64 min (curves 2–8). (d) Rhodopsin (curve 1) was successively irradiated with the orange light for a total of 1, 2, 4, 8, 16, 32, and 64 min (curves 2–8).

(Zorbax SIL column from Du Pont).

Preparation of Frog Rod Outer Segment Membranes. All the manipulations were done under infrared light (>800 nm) by use of darkroom goggles (type 5156, N.V.Optische, Delft, Holland). Frog rod outer segment membranes were prepared from retinas of 12-h dark-adapted frogs (*Rana catesbeiana*) by a 43% (w/w) sucrose flotation method (Fukada et al., 1981) and then suspended in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 8.0) containing 1 mM $MgCl_2$ and 2 mM dithiothreitol (dark-adapted frog rod outer segment membranes). This preparation has all the components necessary for the photoactivation of cGMP phosphodiesterase in the presence of GTP.

Assays. Phosphodiesterase activity was assayed by the use of a reaction mixture composed of 4 mM [^{3}H]cGMP (1.5 Ci/mol), 20 μM GTP, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.0). The enzyme reaction was started by mixing the reaction mixture (10 μL) with a membrane suspension (10 μL; containing 15–30 μg of membrane protein) and then incubating it for 2 min at 30 °C. The reaction was stopped by immersing the reaction tube in boiling water (90 °C) for 1 min. The product of the enzyme reaction, 5′-GMP, was isolated from the reaction mixture by inorganic salt coprecipitation or by thin-layer chromatography on poly(ethylenimine)–cellulose as previously described (Fukada et al., 1981).

The concentration of protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Absorption and circular dichroism (CD) spectra were measured on a Hitachi 330 type spectrophotometer and a JASCO J-20 type spectropolarimeter, respectively.

Results

Binding of Ret5 to Cattle Opsin. The time course of the binding reaction of Ret5 to cattle opsin solubilized in digitonin was followed by monitoring both the absorbance change at 400 nm due to decrease of Ret5 and that at 500 nm due to the concomitant formation of Rh5. Under the condition that a 5-fold excess of Ret5 was incubated with cattle opsin, Rh5 was slowly produced in accordance with pseudo-first-order kinetics with a kinetic constant of 0.0336 M^{-1} s^{-1} in 10 mM HEPES buffer (pH 7.0) containing 2% digitonin at 20 °C. The formation of 11-*cis*-rhodopsin under the same conditions gave a pseudo-first-order kinetic constant of 21.8 M^{-1} s^{-1}. When 11-*cis*-retinal was added to the final mixture in the binding experiment of Ret5 to cattle opsin, only a small amount of rhodopsin (corresponding to 5–10% of the total content of opsin) was additionally formed. This result indicates that Ret5 probably occupies the same binding site of opsin as 11-*cis*-retinal does (retinal-binding site). The large difference in the kinetic constant suggests that Ret5 fits into the retinal-binding site of opsin less well than 11-*cis*-retinal.

It must be marked, incidentally, that Ret5 scarcely bound to cattle opsin either in the membrane or when solubilized with detergents other than digitonin (CHAPS, sucrose lauryl monoester, etc.). Moreover, it bound to frog opsin neither in the membrane nor in detergent solution. In these cases, the decomposition of Ret5 was significantly accelerated in incubation at 20 °C judged from a decrease in absorbance. Thus, the cattle opsin solubilized in digitonin was used as a starting material.

Photoreactivity of Rh5 at −196 °C. The equipment used for this study and the general procedure have already been described in detail (Yoshizawa, 1972). Briefly, the purified Rh5 (see Materials and Methods) solubilized with 2% digitonin in 10 mM HEPES (pH 7.0) was mixed with glycerol (1:2 v/v). The mixture was placed in an optical cell (path length 3 mm) that was fixed in a cryostat. A xenon lamp (2 kW, Ushio) was used for irradiation of the sample, and the light at a desired wavelength was isolated by inserting an interference filter (433 nm, half band width = 5 nm) or a cutoff filter (Toshiba) in front of the xenon lamp. The temperature of the sample was monitored with a copper–constantan thermocouple.

Upon irradiation of Rh5 with blue light at 433 nm at −196 °C, no significant spectral change was observed (Figure 2a). In contrast, irradiation of cattle rhodopsin under the same conditions caused a large red shift of the absorption spectrum and then resulted in a photostationary state with an irradiation time of only 4 min (Figure 2c), indicating the formation of bathorhodopsin.

On the other hand, irradiation of Rh5 with orange light at wavelengths longer than 560 nm (>560 nm; Toshiba VO-58) for 64 min brought about little spectral shift to shorter wavelengths (Figure 2b). As a control, the same irradiation (>560 nm) of rhodopsin produced a blue shift of the absorption

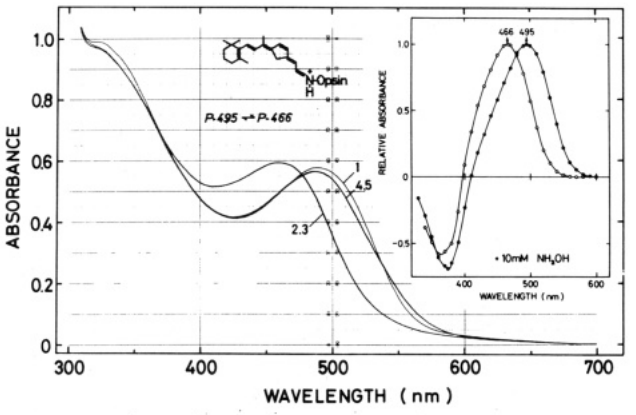

FIGURE 3: Photoreaction of Rh5 at 0 °C. Rh5 (curve 1) was irradiated with an orange light (>560 nm) for 60 and 120 min (curves 2 and 3) and then with a blue light (at 433 nm) for 15 and 30 min (curves 4 and 5). In the insert are shown the difference absorption spectra between before and after thermal bleaching of Rh5 (curve 1 corresponds to ●) and its hypsochromic photoproduct (curve 3 corresponds to ○) as described in the text in detail. They are normalized at the absorption maxima. The ratio in absorbance at maxima (P-466/P-495) was 0.82.

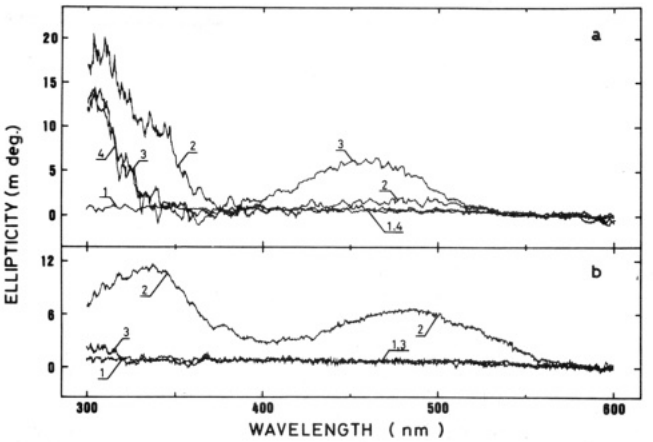

FIGURE 4: CD spectra of Rh5 and rhodopsin. All spectra were measured at 20 °C in cuvettes with a 1-cm optical path. Curve 1 in either (a) or (b) represents a baseline of H_2O. (a) Rh5 (curve 2) was irradiated at 0 °C with orange light (>560 nm) for 120 min to produce P-466 (curve 3). Then, it was thermally bleached at 70 °C for 1 min (curve 4). Difference absorbances before and after the thermal bleaching were 0.79 at 495 nm in the form of P-495 and 0.65 at 466 nm in the form of P-466. (b) Rhodopsin (curve 2) was thermally bleached at 70 °C for 1 min (curve 3). Difference absorbance of the sample before and after the thermal bleaching was 0.79 at 498 nm.

spectrum, indicating the formation of isorhodopsin (9-*cis*-rhodopsin). After 16-min irradiation, a photostationary state was established (Figure 2d).

Then, the sample that had been irradiated with either the orange light (>560 nm) or the blue light (at 433 nm) at −196 °C was warmed to 0 °C. The spectrum thus measured at 0 °C was in complete agreement with that of the original Rh5 (data not shown). This result ruled out the possibility that a labile photoproduct might be formed at −196 °C that was isochromic with Rh5. The lack of photochemical reaction of Rh5 at −196 °C suggests that the cis–trans isomerization is an essential step for the formation of bathorhodopsin.

Photoreactivity of Rh5 at 0 °C. Contrary to our expectations, Rh5 displayed a photoreactivity at 0 °C. As shown in Figure 3, irradiation of Rh5 (Figure 3, curve 1) with an orange light (>560 nm) caused a large blue shift of the absorption spectrum, reaching a photostationary state within 60 min of irradiation (Figure 3, curves 2 and 3), owing to formation of a hypsochromic photoproduct.

In order to estimate the absorption maximum of the photoproduct, hydroxylamine (pH 7.0) at a final concentration of 10 mM was added at 0 °C to both the original Rh5 and its hypsochromic photoproduct, and then they were kept in the dark for 10 min at 0 °C. Under these conditions, they were perfectly stable, because the half-time for decomposition of each component was about 30 min at 21 °C and 14–16 h at 0 °C. In fact, we measured the absorption spectra of them twice successively (4-min interval) and confirmed that the first spectrum was perfectly superimposable on the second one in both cases. After that, the samples were heated to 70 °C for 1 min and rapidly cooled to 0 °C to decompose them presumably into Ret5 oxime and opsin. After it was confirmed that another 1-min heating at 70 °C caused no further spectral change, the difference absorption spectra before and after the thermal bleaching were calculated. The original Rh5 and its hypsochromic photoproduct had their absorption maxima at 495[2] and 466 nm, respectively (inserted of Figure 3). In this paper, we provisionally called the former P-495 and the latter P-466.

[2] The absorption maximum was previously estimated to be at 498 nm from the difference spectrum between the original Rh5 and its thermally bleached product in the absence of hydroxylamine (Ito et al., 1982).

Moreover, irradiation of P-466 with blue light (at 433 nm) at 0 °C resulted in the formation of P-495-like pigment, the absorption spectrum of which was close to that of P-495 but a little broader in band width than that of P-495 (Figure 3, curves 4 and 5). There was a photoreversibility between P-466 and this "broad" P-495. During the photoreactions of Rh5 and its photoproducts, no hydroxylamine was added to the sample, because the presence of 10 mM hydroxylamine caused some decomposition of their chromophores.

Isomeric Compositions of P-495 and P-466. The photoreaction of Rh5 at 0 °C suggested that a change of isomeric composition in the chromophore might be involved. This was because the λ_{max} of P-466 was close to that of 7-*cis*,11-*cis*-rhodopsin (465 nm; Kini et al., 1979). In order to check this possibility, the isomeric composition of the chromophores of P-495 and its photoderivatives at 0 °C were measured, in which synthetic isomers of Ret5 were used as authentic samples for identification of the peaks in chromatographic patterns.

The chromophore of P-495 (corresponding to curve 1 in Figure 3) was composed of 92 ± 5% Ret5 and 8 ± 5% 11-cis,13-cis isomer (mean ± SD of five extractions), and that of P-466 (corresponding to curve 3 in Figure 3) was composed of 84 ± 5% Ret5 and 16 ± 4% 11-cis,13-cis isomer. The chromophore of the broad P-495 (corresponding to curve 5 in Figure 3) consisted of 73 ± 8% Ret5, 11 ± 4% 9-cis,11-cis isomer, and 16 ± 8% 11-cis,13-cis isomer. No 7-cis,11-cis isomer was detected throughout the experiments. Since the content of the 11-cis,13-cis isomer (8%) detected in the original P-495 was probably due to a dark isomerization of Ret5 during the incubation with opsin, it was subtracted from the isomeric compositions of all cases. Then, the content of the 11-cis,13-cis isomer in the chromophore of P-466 was less than 10%, which was too small to characterize the λ_{max} of P-466, 30 nm shorter than that of P-495. The difference in absorption spectra between the original P-495 and the broad P-495 might reflect some new involvement of the 9-cis,11-cis isomer, although the λ_{max} of 9-*cis*,11-*cis*-rhodopsin is a shorter wavelength (480 nm; Kini et al., 1980) than that of rhodopsin.

CD Spectra of P-495 and P-466. Figure 4 shows CD spectra of the original R-5 (P-495), P-466, and cattle rho-

dopsin. Compared to the CD spectrum of rhodopsin, P-495 has a very small α-band in the visible region and comparable β-band in the near-ultraviolet region. This fact indicates that the α-band of the CD spectrum of rhodopsin is induced by a twisted conformation around the C_{12}–C_{13} single bond of the chromophore.

The conversion of P-495 to P-466 shifted the maximum of the CD spectrum in the visible region from about 490 to 460 nm with a remarkable increase in ellipticity, while the CD spectrum in the near-ultraviolet region decreased during this conversion. The thermally bleached sample of the P-466 displayed almost the same CD spectrum in the near-ultraviolet region at P-466 did. This fact suggests that P-466 has almost no β-band that was originated from the chromophore of P-466. Thus, P-466 has only α-band, which was almost comparable to that of rhodopsin in ellipticity normalized with respect to absorption maximum.

Since free Ret5 or its Schiff-base with *n*-butylamine in both unprotonated and protonated forms shows no optical activity in methanol in the region from 300 to 700 nm (data not shown), the apparent CD band of P-466 and its thermally bleached product near 300 nm (Figure 4a, curves 3 and 4) might be attributed to the following two origins. The first possible origin may be the amino acid residue(s) (Trp?) of proteins in the sample. In the present experiment, the protein concentration of the Rh5 sample was much higher than that of cattle rhodopsin sample. Thus, it seems reasonable that a small but clear CD band near 300 nm displayed by the thermally bleached sample of rhodopsin (difference between curves 1 and 3 in Figure 4b) might be measured as a strong band in P-466 and its thermally bleached product. The origin of this CD band is not clear whether it is due to amino acid residue of opsin or some impurity in the sample. The second possibility may be an artifact that is sometimes recorded in measuring a sample with a high absorbance. The absorbance of P-466 or its thermally bleached product at 300 nm was above 2.0 in this experiment.

Effects of P-495 and P-466 on cGMP Phosphodiesterase. Activation abilities of Rh5 and its photoderivatives on the enzyme in frog rod outer segment membranes were examined by a mixing experiment as follows. Dark-adapted frog rod outer segment membranes containing rhodopsin, GTP-binding protein, and cGMP phosphodiesterase were prepared in the complete dark condition. They were used as an enzyme source because they showed high activation ability of phosphodiesterase (Fukada et al., 1981). First, by the mixing of cattle opsin or rhodopsin (prepared as described under Materials and Methods) with dark-adapted frog rod outer segment membranes in the dark, it was confirmed that cattle opsin or rhodopsin induced no enzyme activation of the membranes [(■) or (▲) in Figure 5]. Next, the cattle rhodopsin was irradiated with orange light (>560 nm) at 0 °C for 10 min to produce a bleaching intermediate composed of mainly metarhodopsin II and then mixed with the dark-adapted frog rod outer segment membranes. This mixing induced a remarkable activation of phosphodiesterase in the membranes [(△) in Figure 5], which indicates that cattle and frog rhodopsins are common in activation abilities on the enzyme of frog rod outer segment membranes. On the basis of these control experiments, Rh5 and its photoderivatives were mixed with the dark-adapted frog rod outer segment membranes to measure their activation abilities on the enzyme. As shown in Figure 5, Rh5 [(●), corresponding to curve 1 in Figure 3], P-466 [(◑), corresponding to curve 3 in Figure 3], or broad P-495 [(○), corresponding to curve 5 in Figure 3] brought about no enzyme activation. A slight increase in the enzyme activation abilities with increase of the concentration of the samples, which were comparable to those of rhodopsin (▲) and opsin (■), may be due to some effect of digitonin used as a solubilizer on the enzyme in the membranes. Therefore, Rh5 and its photoderivatives should be regarded as physiologically inactive species, provided that the activation ability on cGMP phosphodiesterase is a measure of physiological activity of a rhodopsin analogue.

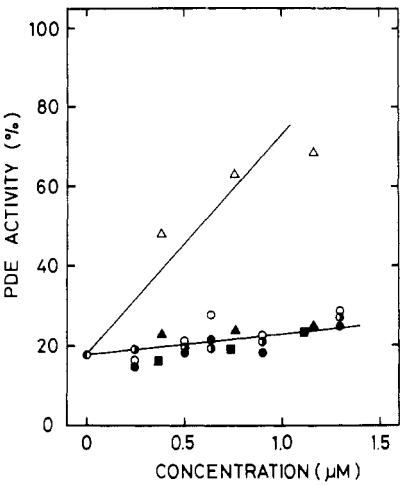

FIGURE 5: Effect of Rh5 on cGMP phosphodiesterase in frog rod outer segment membranes. Cattle opsin (■), Rh5 (P-495 corresponding to curve 1 in Figure 3; ●), P-466 (curve 3 in Figure 3; ◑), broad P-495 (curve 5 in Figure 3; ○), rhodopsin (▲), or photoproduct of rhodopsin that had been irradiated with orange light (>560 nm) at 0 °C for 10 min (△) was appropriately diluted with 10 mM HEPES buffer (pH 7.0) and mixed with 9 volumes of dark-adapted frog rod outer segment membrane suspension (1:9 mixture). Then, the phosphodiesterase activity was measured in the dark at 30 °C for 2 min. The activity was plotted against the concentration of cattle opsin, Rh5, or rhodopsin in the 1:9 mixture. The activities were expressed as percent of that of irradiated (>560 nm, at 0 °C for 1 min) frog rod outer segment membrane suspension. One-hundred percent corresponds to 0.95 ± 0.07 μmol of cGMP hydrolyzed min^{-1} (mg of protein)$^{-1}$.

Discussion

The study of Rh5 by low-temperature spectrophotometry showed that the isomerization around the C_{11}–C_{12} double bond of the retinylidene chromophore of rhodopsin was an essential step for formation of bathorhodopsin. This fact coincides with the result of Mao et al. (1981) using an analogue locked 11-*cis* bond with a seven-membered ring. At 0 °C, however, Rh5 exhibited a photochemical reaction, while the seven-membered analogue did not at 20.8 °C (Mao et al., 1981). Another difference between these two analogues was observed in the CD spectrum. Close similarity in the CD spectrum between rhodopsin and the seven-membered rhodopsin (Akita et al., 1980) should be ascribed to a constrained nonplanar shape of the chromophore. Because Rh5 retains a planar configuration from C_9 to C_{14} in the side-chain moiety of its chromophore (Figure 1) (Ito et al., 1982), it showed only a small α-band in its CD spectrum (Curve 2 in Figure 4a). This observation demonstrates that the optical activity of rhodopsin in the visible region originates from a twisted conformation around the C_{12}–C_{13} single bond in the retinylidene chromophore. Furthermore, the slow rate of the binding reaction of Ret5 to cattle opsin in comparison with that of the regeneration of rhodopsin suggests a selective binding of opsin to a twisted 11-*cis*-retinal in 12-s transoid form. The planar and rigid conformation of the side chain of Ret5 might be inconvenient for entering the retinal-binding site of opsin. On the other hand, the β-band

in the CD spectrum of Rh5 was roughly equal to that of rhodopsin in difference ellipticity between the original pigment and its thermally bleached product (Figure 4). Since the β-band of the CD spectrum of rhodopsin originates from a twisted conformation around the C_6–C_7 single bond (Shichida et al., 1978), the equivalence in the difference ellipticity suggests that the side-chain moiety between C_7 and C_{12} of the five-membered retinylidene chromophore is twisted out of the plane of the β-ionone ring in direction and angle similar to that of rhodopsin.

P-466 has almost no β-band in its CD spectrum (curve 3 in Figure 4a). This fact suggests that the photoconversion of P-495 to P-466 may diminish the twist at the C_6–C_7 single bond to some extent. The α-band of the CD spectrum of P-466 (curve 3 in Figure 4a), however, which is comparable in ellipticity to that of rhodopsin (curve 2 in Figure 4b) suggests the presence of an asymmetric conformation in the side-chain moiety of the chromophore of P-466. This conformation is probably due to a twist at a single bond, because it is easier than that at a double bond, and also a distortion around a double bond may cause a bathochromic shift in the absorption spectrum (Kakitani & Kakitani, 1975). The twisted single bond may be either the C_8–C_9 or the C_{14}–C_{15} single bond, because no other single bond in the side chain moiety can be distorted, judging from the chemical structure of Ret5 (Figure 1; Ito et al., 1982). The conformation of the chromophore of P-466 may be speculated to be twisted around the C_8–C_9 single bond rather than around the C_{14}–C_{15} single bond for the following reasons. The fact that P-495 shows no photoreactivity at −196 °C indicates that the mode of conformational change of the chromophore in the course of photoconversion of P-495 to P-466 should not be similar to that in the course of conversion of rhodopsin to bathorhodopsin. In the latter case, the chromophore of rhodopsin is isomerized from 11-cis form to an all-trans one in the way that the region of C_{13} to Schiff-base nitrogen of the chromophore concomitantly with the lysine residue moves spatially in the chromophore-binding site. Thus, even at liquid nitrogen temperature, the chromophore-binding site should have a space enough for rotation around the C_{14}–C_{15} single bond. Therefore, the fact that P-466 was not produced at liquid nitrogen temperature suggests that the formation of P-466 from P-495 is due not to the twisting around the C_{14}–C_{15} single bond but to that around the C_8–C_9 single bond. In fact, irradiation of rhodopsin above −70 °C produced a photoproduct that has 7-*cis*-retinal as its chromophore (Maeda et al., 1978), suggesting that the chromophore-binding site above this temperature became flexible enough to accommodate the twisted chromophore around the C_8–C_9 single bond. Furthermore, the twist around the C_8–C_9 single bond may assist in increasing the coplanarity between the plane of the β-ionone ring and that of the C_7–C_8 double bond, resulting in the decrease of the β-band of the CD spectrum in the course of the photoconversion of P-495 to P-466. The twist around the C_{14}–C_{15} single bond, however, would not induce an increase in the coplanarity on the assumption that the β-ionone ring of the chromophore is rigidly fixed in the β-ionone ring binding site of opsin (Matsumoto & Yoshizawa, 1975). On the basis of these speculations, the conformations of the chromophores of P-495 and P-466 were schematically drawn as in Figure 6.

The degree of the secondary interaction between opsin and the protonated retinylidene chromophore was expressed in a term of an "opsin shift", which was defined by the difference (in cm^{-1}) in absorption maximum between the protonated Schiff base of retinal or its analogue with *n*-butylamine and

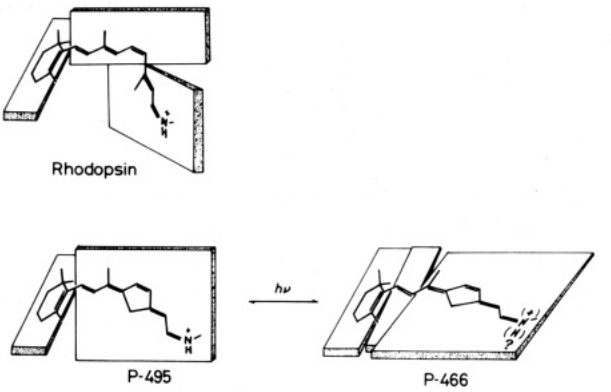

FIGURE 6: Schematic models for the conformations of chromophores of P-495 and P-466. As a reference, a model for the conformation of the chromophore of rhodopsin is concurrently drawn according to Honig et al. (1979).

the pigment derived from it (Nakanishi et al., 1979). From the analysis of the opsin shifts in a series of dihydrorhodopsin systems, they proposed an external point-charge model in which the Schiff base was protonated and had a counterion at 3-Å distance, and the second charge was located at about 3 Å from both C_{12} and C_{14} of the retinal side-chain moiety (Honig et al., 1979). In the Rh5 system, the opsin shift was 1350 cm^{-1} for P-495, which was half of that of rhodopsin, 2700 cm^{-1} (Nakanishi et al., 1979). This small opsin shift indicates that the interactions between the chromophore and two negative charges of opsin are remarkably different from those in the case of rhodopsin. According to Honig's calculation (Honig et al., 1979), this reduction of opsin shift would be expected in the following cases. (i) The chromophore of P-495 should be less than 3 Å distant from the first negative charge (which may interact with a proton at the Schiff base). (ii) The chromophore of P-495 should be more than 3 Å distant from the second negative charge (which is located near the C_{12} and C_{14} positions of the chromophore), owing to the planarity of the side-chain moiety. The fact that the λ_{max} of P-466 is farily close to that of the protonated Schiff base of Ret5 with *n*-butylamine (464 nm) may imply that the second negative charge may be far enough not to interact with the chromophore in P-466.

We may suppose that, as shown in Figure 6, the angle between the planes of the β-ionone ring and the side chain in the chromophore of Rh5 changes remarkably in the course of photoconversion of P-495 to P-466; this change might keep the distance away from the chromophore to the second negative charge on the assumption that the β-ionone ring of the chromophore is rigidly fixed in the β-ionone ring binding site of opsin. Another explanation for the blue shift in P-466 is that the chromophore of P-466 may be less than 3 Å distant from the first negative charge. It is difficult for us to judge either (or both) of them is true in this case within our experimental data, and it should be confirmed by other methods.

At last, it was confirmed that Rh5 and its photoderivatives were inactive physiologically on the assumption that cGMP phosphodiesterase activation is involved in the visual transduction process in a rod cell. This observation confirmed the view that the isomerization of the retinylidene chromophore of 11-cis to an all-trans form was the essential step for the phototransduction process. Furthermore, since the distance between the center of the β-ionone ring and the Schiff-base nitrogen of the chromophore (longitudinal length of the chromophore; Matsumoto & Yoshizawa, 1978) of Rh5 does not change remarkably in the course of photoconversion between P-495 and P-466, the results are consistent with our

previous model (Fukada et al., 1982; Yoshizawa & Fukada, 1983) in which we have proposed that an "expanded" conformation around the retinal-binding site of opsin should be necessary for activation of the enzyme.

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Registry No. Ret5, 83631-39-4; 11-*cis*-retinal, 564-87-4.

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