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Mechanism of Isomerization of Rhodopsin Studied by Use of 11-Cis-Locked Rhodopsin Analogues Excited with a Picosecond Laser Pulse†

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ABSTRACT: Primary photochemical behaviors of cattle rhodopsin analogues (Rh5 and Rh7) having cyclopenta- and cycloheptatrienylidene 11-cis-locked retinals (Ret5 and Ret7, respectively) were studied by excitation with a picosecond laser pulse (wavelength 532 nm; duration 21 ps). Picosecond absorption and fluorescence measurements of Rh5 showed formation of only a long-lived excited singlet state ($\tau_{1/e}$ = 85 ps). The excited state of the retinal analogue having a five-membered ring was stabilized in protein (Rh5) more than in solvent (protonated Schiff base of Ret5; PSB5). Excitation of Rh7 produced two ground-state photoproducts, Rh7(580) and Rh7(630). According to the analysis of photon density dependency, Rh7(580) was a single-photon product of Rh7, while Rh7(630) was the photoproduct of Rh7(580). Fluorescence emitted from a seven-membered ring system like Rh7 or a protonated Schiff base of Ret7 (PSB7) was weaker than that in a corresponding five-membered ring system, especially in protein (Rh7). The difference in photoreaction between Rh5 and Rh7 may originate from the difference in fixation of the 11-cis form. On the basis of the spectral and kinetic similarities between Rh7(580) and photorhodopsin, a precursor of bathorhodopsin, it was proposed that both have twisted all-trans chromophores in the way of the isomerization. The protein moiety of rhodopsin which fixes the chromophore at both ends seems to accelerate the rotation of the C_{11}–C_{12} double bond and to prevent it from going through relaxation processes other than the isomerization. This may be a plausible reason why rhodopsin has a large quantum yield (0.67).

Absorption of a photon by rhodopsin isomerizes its 11-*cis*-retinylidene chromophore to all-trans form. The photoisomerization takes place at extremely high speed so that rhodopsin has a high photosensitivity. Until recently, it was believed that bathorhodopsin would be the earliest intermediate of photobleaching of rhodopsin. Since bathorhodopsin has a twisted *all-trans*-retinal as its chromophore (Yoshizawa & Wald, 1963; Yoshizawa & Horiuchi, 1972; Fukada et al., 1984; Eyring et al., 1982), it was recognized that the isomerization of the chromophore began with a Franck–Condon state and was completed at the ground state via an excited common state of rhodopson and bathorhodopsin.

Several years ago, Shichida et al. (1984) detected an earlier intermediate than bathorhodopsin, called photorhodopsin. This intermediate has its absorption maximum at a longer wavelength than that of bathorhodopsin. What is the structure of the chromophore in photorhodopsin? Is the chromophore of photorhodopsin an intermediate on the way to the isomerization? A powerful tool to solve this question is to use some retinal analogues in which the isomerization of the C_{11}–C_{12} double bond is locked. Two kinds of analogues have been synthesized; one is locked by a five-membered ring (Ret5; Ito

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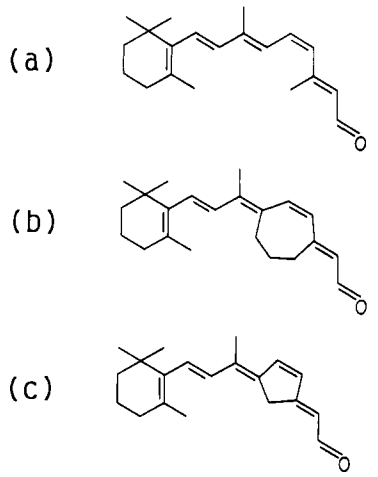

FIGURE 1: Structural formula of (a) 11-*cis*-retinal, (b) seven-membered retinal, and (c) five-membered retinal.

et al., 1982) and the other by a seven-membered ring (Ret7; Akita et al., 1980) (Figure 1). By use of the rhodopsin analogues having these retinal analogues as their chromophores, it was inferred that the chromophore of bathorhodopsin is already isomerized to the all-trans form (Mao et al., 1981; Fukada et al., 1984).

It should be noted that these retinal analogues differ from each other in the manner of prohibition of the isomerization around the 11-ene. Namely, Rh5 has a planar rigid ring, whereas Rh7 has a relatively flexible ring which enables the 11-ene or the nearby single bonds to distort to some extent. The fact that Rh7 has a CD spectrum similar to that of rhodopsin (Akita et al., 1981) while Rh5 has a negligible CD in the α-band region (Fukada et al., 1984) supports these aspects. Therefore, we expected to secure some information regarding the configuration and/or conformation of the chromophore of photorhodopsin by comparing the primary processes of Rh5 and Rh7.

Some years ago, Buchert et al. (1983) carried out a picosecond laser photolytic experiment on Rh7 and found two transient photoproducts in the picosecond time scale. Though these products were identified as excited-state species, the present work indicates that one of these photoproducts is likely to be a ground-state species and the other its photoproduct. On the other hand, excitation of Rh5 only produced an excited state from which a stimulated emission signal could be observed.

Materials and Methods

Preparation of Cattle Rod Outer Segments. Cattle rod outer segments (ROS) were isolated from fresh retinas by a conventional sucrose stepwise flotation method (Shichida et al., 1987) and then suspended in 10 mM Hepes buffer (pH 7.0). The ROS suspension was irradiated in the presence of 100 mM hydroxylamine with a yellow light from a 1-kW projector lamp (Sanko) passing through a Toshiba VO-54 cutoff filter for complete bleaching of rhodopsin. The ROS thus obtained was washed seven times with the Hepes buffer and once with distilled water to remove unreacted hydroxylamine and peripheral protein. After lyophilization, the ROS was washed five times with petroleum ether to remove retinal oxime and partly lipids and then suspended in the Hepes buffer.

Preparation of Rh5. Ret5 was synthesized by the method described previously (Ito et al., 1982) and incorporated into cattle opsin according to a method reported previously (Fukada et al., 1984). Opsin in the ROS prepared above was solubilized with 2% digitonin dissolved in the Hepes buffer and then diluted into 1% digitonin solution, followed by mixing with a 2.2-fold molar excess of Ret5 dissolved in ethanol ($^1/_{100}$ volume of the digitonin solution) and incubating for 24 h at 20 °C to generate Rh5. In order to remove the unreacted Ret5, the preparation containing Rh5 was applied to a concanavalin A–Sepharose 4B affinity column, which had been equilibrated with 10 mM Hepes buffer (pH 7.0) supplemented with 1% digitonin, 67 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. After thorough washing of the column bed with the buffer, Rh5 was eluted with the buffer further supplemented with 200 mM methyl α-D-mannopyranoside. After removal of the methyl α-D-mannopyranoside and other ions in the eluate by dialysis, Rh5 was concentrated by means of flow dialysis with an ultrafiltration membrane (YM-30, Amicon). Absorbance of the sample at 532 nm was then adjusted to be 0.6 for absorption measurements or 0.3 for fluorescence measurements (2-mm light path).

Preparation of Rh7. Ret7 was synthesized by the method described previously (Akita et al., 1980). The ROS suspension obtained above was mixed with about a 5-fold excess of Ret7 dissolved in ethanol and incubated for 24 h at 20 °C to produce Rh7. After being washed seven times with 10 mM Hepes buffer (pH 7.0) and once with distilled water, the ROS was lyophilized, treated with petroleum ether, and solubilized in 2% digitonin dissolved in the buffer. Absorbance of the extract at 532 nm was then adjusted to be 0.7 for absorption measurements or 0.3 for fluorescence measurements (2-mm light path).

Preparations of PSB5 and PSB7. Protonated Schiff bases of Ret5 (PSB5) and Ret7 (PSB7) were prepared as previously described (Fukada et al., 1984). Each retinal analogue (10 nmol) dissolved in hexane was dried under a N_2 stream, to which 0.5 mL of *n*-butylamine was added. This mixture was sealed under a N_2 atmosphere into a reaction tube, which was shaken vigorously and kept on ice for 3 h in the dark. The sample was then dried under a N_2 stream and dissolved in 1 mL of methanol. The Schiff bases thus formed were protonated by addition of 2.5 μL of 10 N HCl. The absorption maxima of PSB5 and PSB7 shifted from 353 to 464 nm and from 355 to 437 nm, respectively. Absorbance of each sample at 532 nm was adjusted to be 0.3 (2-mm light path).

Laser System for Excitation of the Samples. A mode-locked Nd^{3+}:YAG laser (Matsui) was used as a light source of the excitation pulse (Shichida et al., 1984). The fundamental pulse from the laser was converted to its second harmonic (wavelength 532 nm) by being passed through a phase-matched KDP crystal. The pulse width was measured by a streak camera (Hamamatsu Photonics) to be 21 ps. The energy of the pulse was monitored by a biplanar photodiode (Hamamatsu Photonics) in each excitation and calibrated by a joule meter (Gentec) at the end of experiments. The energy for excitation of the sample could be varied up to about 300 μJ/1.8 mmϕ in the experiments.

Optical System for Measurement of Picosecond Absorption Spectra. The optical system was a double-beam picosecond spectrometer linked with the Nd^{3+}:YAG laser (Shichida et al., 1984). A sample in an optical cell (2-mm light path) was excited by the single green pulse described above. A cutoff filter (VO-54 or -55, Toshiba) was used for reduction of scattering light due to the excitation pulse. Consequently, the transient absorption spectrum was measured only at wavelengths longer than 550 nm. A picosecond continuum for the probe pulse was generated by focusing the fundamental pulse (25 mJ) into a glass block. The probe pulse was split into two

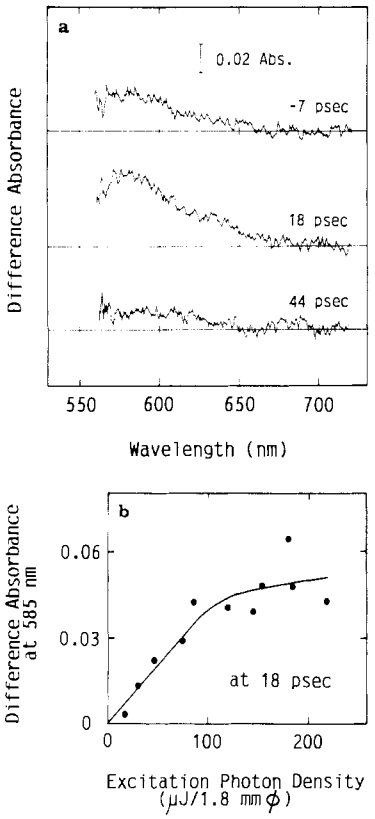

FIGURE 2: (a) Difference absorption spectra before and after excitation of Rh7 with a single green pulse (wavelength 532 nm; pulse width 21 ps; excitation energy 121 μJ/1.8 mmϕ) at room temperature. The spectra corresponded to difference spectra between Rh7 and its phototransients, which were measured at −7, 18, and 44 ps after excitation. (b) Dependency of absorbance change of Rh7 at 585 nm (an average of 35 points in the range between 578 and 592 nm) on photon density of the single green pulse (532 nm). The absorbance was measured at 18 ps after excitation of Rh7 with the green pulse.

beams by a half-mirror; one was passed through the sample (I^{sam}), while the other, used for a reference pulse, was not (I^{ref}). The probe pulses were focused on a slit of a polychromator (Jarrell Ash) and detected by a Vidicon detector (ISIT, PAR). Each experiment was carried out as follows: First, light intensities of probe pulses without excitation of sample were measured (I_1). Second, intensities of probe pulses after excitation of the sample were measured (I_2), and, finally, those of scattered excitation light or of luminescence emitted from the sample due to the excitation were measured without the probe pulse (I_3). Thus, the transient absorption spectra were calculated with

$$\Delta A = \log \frac{I_1^{sam}}{I_1^{ref}} - \log \frac{I_2^{sam} - I_3^{sam}}{I_2^{ref} - I_3^{ref}} \quad (1)$$

If neither scattering light nor luminescence is generated from the sample, I_3^{sam} and I_3^{ref} in the second term should be zero.

The sample in the optical cell was renewed after several excitations, even though no absorbance changes were detected. The transient absorption spectra thus obtained were accumulated and analyzed by an OMA 2 system (PAR).

Optical System for Measurement of Picosecond Fluorescence Kinetics. The single green pulse was used for excitation of the sample in an optical cell (2-mm light path). The spot size of the excitation pulse was 2.0 mm^2 at the sample position after passing an aperture. The fluorescence from the sample was collected onto a 30-μm entrance slit of a streak camera system (C-2000, Hamamatsu Photonics). A 560-nm cutoff filter (Toshiba) was placed in the fluorescence path to eliminate any scattered light owing to the excitation pulse. The time resolution (FWHM) of the whole apparatus was 25 ps. To determine the decay time of fluorescence, least-squares fitting procedures were used with deconvolution of the time resolution of the system. The sample in the optical cell was renewed after several excitations, even though no absorbance changes were detected. Both absorption and fluorescence measurements were performed at 18 °C.

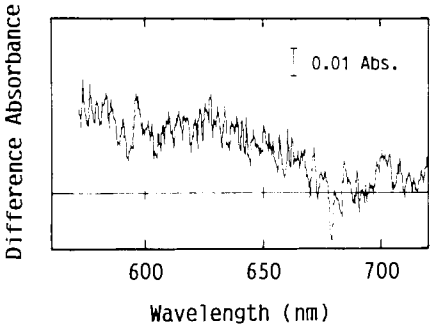

FIGURE 3: Difference absorption spectrum before and after excitation of Rh7 with an intense green pulse. The spectral change was measured at 44 ps after the excitation with a green pulse (532 nm) of 240 μJ/1.8 mmϕ. The excitation photon density was calculated to be 2.1 photons per molecule.

RESULTS

Picosecond Absorption Spectra of Rh7 and Rh5. Figure 2a shows transient absorption spectra (difference spectra between Rh7 and its transients) measured at three different times after the excitation of Rh7 with the 532-nm laser pulse. Since the pulse widths of both the probe and the excitation pulses were 21 ps, the transient absorbance changes could be observed at 7 ps before the peak of the excitation pulse reached the sample (−7 ps after the excitation). An increase in absorbance at wavelengths longer than 550 nm was observed, indicating that a photoproduct having absorption maximum at longer wavelengths than that of the original pigment (λ_{max} 490 nm) was produced. Since the difference absorption maximum is located at about 580 nm, we shall call this product Rh7(580). The difference spectrum measured 18 ps after excitation was similar in shape to that measured at −7 ps after excitation. However, the positive absorbance at these wavelengths mostly disappeared 44 ps after excitation. This fact indicated that Rh7(580) decayed directly to the original pigment within 44 ps. As shown in Figure 2b, a linear relationship between the photon density and the increase in absorbance was observed up to 70 μJ/1.8 mmϕ, indicating that Rh7(580) was directly produced from the original pigment, Rh7, by a one-photon absorption process. Compared to Rh, Rh7 was very insensitive to light, because the difference absorbance in Figure 2b was 0.008 at 20 μJ/1.8 mmϕ while that at 15 ps after excitation of Rh was 0.05 at 20 μJ/1.8 mmϕ (Matuoka et al., 1984).

Under the experimental conditions described above, we failed to detect any formation of Rh7(640) (a photoproduct having absorption maximum at about 640 nm), which was reported to be produced from Rh7(570) with a time constant of 20 ps (Buchert et al., 1983). Accordingly, the photon density of the excitation pulse was raised so that Rh7 molecule in the sample could absorb about two photons (2.1 photons/rhodopsin). Under such condition, a photoproduct having a difference absorption maximum at about 630 nm [Rh7(630)] could be detected (Figure 3). The results clearly show that Rh7(630) is generated only by a multiphoton reaction of the original pigment. The fact that the formation of Rh7(630) was observed even 40 ps after excitation indicated that its

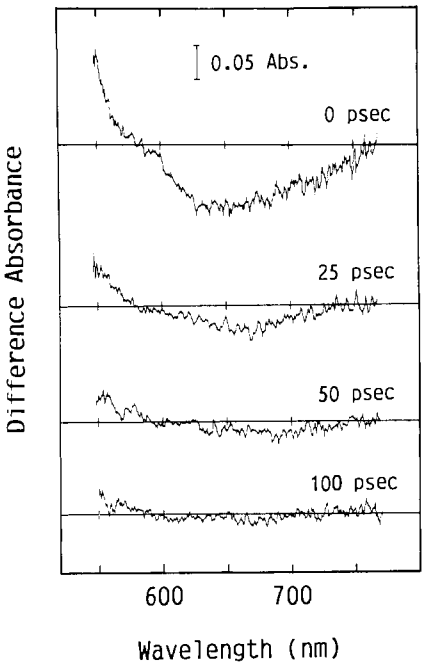

FIGURE 4: Difference spectra before and after excitation of Rh5 with the green pulse (532 nm, 21 ps, 20 μJ/1.8 mmϕ). The spectra were measured at 0, 25, 50, and 100 ps after excitation. Since Rh5 has no absorption in the wavelength range longer than 600 nm, the negative difference absorption in this region indicates a stimulated emission signal of the excited singlet state of Rh5 by the probe pulse.

decay time constant was larger than that of Rh7(580).

Figure 4 shows the transient spectral changes measured after excitation of Rh5 with the laser pulse, the photon density of which was 20 μJ/1.8 mmϕ. Immediately after excitation (0 ps), the absorbance at about 550 nm increased, while that about 600 nm decreased. This change almost disappeared after 100 ps. Two points should be noted. The first is the positive absorbance change at about 550 nm. This absorbance change is rather large compared with those of Rh and Rh7. That is, the laser-induced changes of difference absorbance of Rh and Rh7 at 20 μJ/1.8 mmϕ were 0.05 (Matuoka et al., 1984) and 0.008 (Figure 2b), respectively, while that of Rh5 was about 0.1 (Figure 4). The second point is the negative absorbance above 600 nm. Since Rh5 has no absorbance above 600 nm, this negative absorbance is due to a stimulated emission signal by the probe pulse. A possibility that it might be due to fluorescence from an excited state of Rh5 was excluded because in our experimental conditions a signal due to the fluorescence should not delay since the detector was kept open throughout the experiments, and the contribution of fluorescence to the difference spectra is negligible according to eq 1.

On the other hand, fluorescence from Rh5 was easily observed by this optical system in which probe pulses were not used (I_3 in eq 1). Figure 5 shows a fluorescence spectrum of Rh5 after correction of the sensitivity of the detector as a function of wavelength. Since a VO-55 cutoff filter was used to eliminate any scattering due to the excitation pulse, the spectrum above 570 nm was reliable. The fluorescence maximum was located at about 620 nm. It should be noted that no fluorescence was observed in the case of Rh or Rh7 (see below) under similar experimental conditions.

Picosecond Fluorescence Kinetics of Rh5, PSB5, Rh7, and PSB7. Figure 6a shows the fluorescence kinetics of Rh5 measured after excitation with the green pulse. The simulation curve (solid line) was calculated so as to fit the experimental data (dotted line) by changing the decay time constants, as-

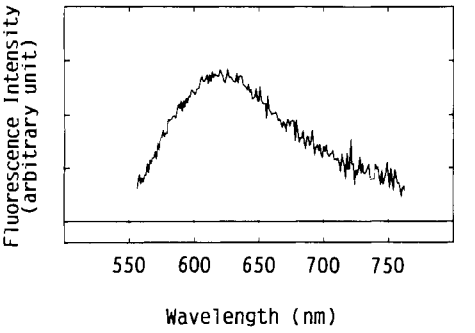

FIGURE 5: Fluorescence spectrum from Rh5 excited with the green pulse. The fluorescence was measured in the setup for measurement of the picosecond absorption spectrum (detector was set in the same direction as the excitation pulse). As VO-55 cutoff filter was used to eliminate scattering; the signal below 570 nm was decreased to some extent. The spectrum shown in this figure is the sum of 15 data. In the measurement of picosecond absorption (Figure 4), this spectrum was subtracted from that of the probe pulse for absorption measurement to eliminate the contribution of the fluorescence (see text).

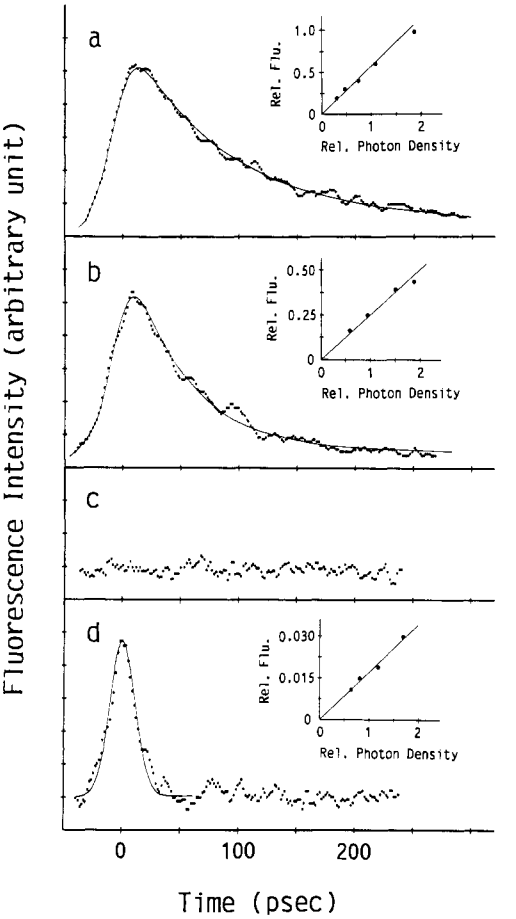

FIGURE 6: Fluorescence kinetics of Rh5 (a), PSB5 (b), Rh7 (c), and PSB7 (d). The intensity of fluorescence emitted from them by excitation with the green pulse (532 nm) was measured above 560 nm at room temperature. No fluorescence was observed from Rh7 (c) as well as from Rh. These are the sum of 5–11 data. The solid lines in (a) and (b) are fitting curves with consideration of the time resolution of the whole apparatus (25 ps). The results of simulations gave $\tau_{1/e}$ = 85 ps in Rh5 (a) and $\tau_{1/e}$ = 50 ps in PSB5 (b). The solid line in (d) indicates the instrumental response function (half-width 25 ps). (Inset) Relationship between fluorescence intensity and excitation photon density of the picosecond laser pulse. Each point is the average of 4–6 data. Each fluorescence signal from Rh5 (a), PSB5 (b), and PSB7 (d) was proportional to the excitation photon density and saturated on more intense excitation. Each sample had the same absorbance at 532 nm (0.3 at 2-mm light path). The ratio among Rh5, PSB5, and PSB7 in the fluorescence signal integrated against the time coordinate was 1, 0.44, and 0.03, respectively.

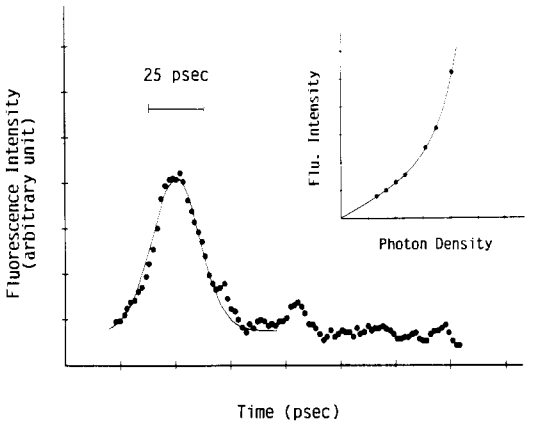

FIGURE 7: Fluorescence kinetics of Rh7. Since no fluorescence was measured by the usual setup (Figure 6), the aperture to determine the spot size of the excitation pulse was replaced by a lens, and the excitation pulse was focused onto the sample. Thermally bleached Rh7 samples displayed no fluorescence. This is the sum of 9 data. The solid line indicates the instrumental response function (half-width 25 ps). (Inset) Relationship between fluorescence intensity and excitation photon density of the picosecond laser pulse. Each point is the average of 4 data.

suming that the instrumental response function could be expressed by Gaussian distribution (half-width 25 ps). The decay time constant of the fluorescence was estimated to be 85 ps. No fluorescence was observed in a thermally bleached Rh5 sample by the green pulse. The excitation photon density dependency of the fluorescence intensity showed a linear relation (Figure 6a, inset).

In the case of PSB5, the decay time constant was 50 ps (Figure 6b). The relation between fluorescence intensity and excitation photon density showed a linear function, and the relative fluorescence intensity of PSB5 compared to that of Rh5 measured above 560 nm was 0.44 (Figure 6b, inset).

With the same optical setup, no fluorescence from Rh7 or Rh was observed (Figure 6c). On the other hand, fluorescence from PSB7 was observed as shown in Figure 6d, where the solid line denoted the response function of our instrument (Gaussian distribution with a half-width of 25 ps). This result indicated that the lifetime of the excited state of PSB7 was too short to be detectable under experimental resolution, even though fluorescence could be observed. The intensity of fluorescence from PSB7 was proportional to the excitation photon density and was small compared with that of Rh5 or PSB5 (Figure 6d, inset).

As described above, an intense excitation of Rh7 induced the generation of Rh7(630), which was similar to Rh7(640) reported by Buchert et al. (1983). Since they observed a fluorescence not from Rh7(570) but from Rh7(640), a strong irradiation of Rh7 may be expected to induce any fluorescence. Thus, the aperture in front of the sample cell was removed, and a lens was set so as to focus an excitation pulse into the sample. This modification markedly increased the photon density at the sample position and resulted in the observation of fluorescence from Rh7 (Figure 7). Thermally bleached Rh7 samples displayed no fluorescence under the excitation conditions. The kinetic behavior of the fluorescence demonstrated a good fit to a Gaussian distribution curve with a half-width of 25 ps. It should be noted that the fluorescence signal from Rh7 was not proportional to the excitation photon density, but the rate of fluorescence increase becomes larger when an intense excitation is used (Figure 7, inset). Since the process of formation of Rh7(580) was related to a one-photon absorption as shown in Figure 2b, the fluorescence observed here originated in another excited state which could not be produced by a single-photon absorption of Rh7.

Discussion

Photochemical Reaction of Rh5. Picosecond absorption and fluorescence measurements of Rh5 clearly demonstrated an excited singlet state of Rh5, the lifetime of which corresponds to the single-exponential decay of the fluorescence of Rh5 ($\tau_{1/e}$ = 85 ps; Figure 6a). Rh5 is about 100 times longer in lifetime of the excited state than Rh (Doukas et al., 1984), probably owing to inhibition of photoisomerization at the center of the chromophore of Rh5. The positive difference absorption in the wavelength region from 550 to 600 nm shown in Figure 4 can be attributed to an absorption of the first excited singlet state (S1 absorption) while the negative absorption in the range from 600 to 750 nm to a stimulated emission by the probe pulse for the absorption measurements. The reason why the decay time course of these absorption changes is slightly shorter than that of the fluorescence is as yet unclear.

This is the first finding of stimulated emission in animal rhodopsin and its analogue systems, though recently some reports described the stimulated emissions from a bacteriorhodopsin analogue (Polland et al., 1984), halorhodopsin (Polland et al., 1985), and bacteriorhodopsin (Dobler et al., 1988; Mathies et al., 1988). Generally, the stimulated emission can be observed when the lifetime of excited state is longer than the pulse duration and the energy difference beteen the excited and ground states is close to the frequency of the probe pulse. An absorption of the excited singlet state has been detected in bacteriorhodopsin (Sharkov et al., 1985; Polland et al., 1986; Petrich et al., 1987) and its analogue (Polland et al., 1984).

It should be noted that an excited singlet state of Rh5 has an absorption maximum at the longer wavelength side like many organic molecules, while the absorption maximum of an excited state of bacteriorhodopsin was located at the shorter wavelength side than that of the original pigment. Polland et al. (1984), however, observed a red-shifted excited singlet state of the bacteriorhodopsin analogue having 9,12-phenylretinal as its chromophore. This chromophore is prohibited to isomerize in protein like the chromophore of Rh5, even though the C_{13}–C_{14} double bond of the phenylretinal could be twisted. On the basis of these facts, we can speculate that the interaction between an isomerizable chromophore and opsin in the excited singlet state is very unique, resulting in a shorter wavelength shift of the absorption maximum. It will be interesting to know where the absorption maximum of an excited state of Rh is located.

Fukada et al. (1984) reported that irradiation of Rh5 at 0 °C yielded a photoproduct (P-466) having an absorption maximum at 466 nm and a positive CD at α-band. Since the quantum yield of formation of P-466 was very low and the sample after excitation with the picosecond pulse displayed no absorbance change, it seems reasonable to assume that the formation of P-466 would be negligible under our experimental conditions.

A noteworthy point is that the decay time of fluorescence from the excited singlet state of Rh5 (85 ps) was longer than that of PSB5 (50 ps). The lifetime of the excited state would depend on a natural radiative lifetime and a competitive nonradiative relaxation process. The natural lifetimes of Rh5 and PSB5 were estimated to be about 4.8 and 5.6 ns, respectively, assuming that the extinction coefficients are similar to those of Rh (40 600; Wald & Brown, 1953) and the protonated Schiff base of 11-*cis*-retinal (26 700; Becker et al., 1985). Both observed lifetimes are considerably shorter than the natural radiative lifetimes. Thus it seems probable that

an internal conversion via a nonradiative process into the ground state is a dominant process for depopulation of the excited state. The fact that the natural radiative lifetime of Rh5 was slightly shorter than that of PSB5 while the observed lifetime of the former was longer than that of the latter suggests that the internal conversion is less in protein than in solution. Since the nonradiative process due to any conformational change in the center of the chromophore would be negligible in both cases, the difference in decay time should be due to the flexibility of both ends of the chromophore that interact with the protein or solvent. In fact, Ret5 in Rh5 is fixed by the protein at both ends, while Ret5 in solution seems to be flexible. Therefore, we believe that the slow rate of internal conversion via a nonradiative process in the protein is due to fixation of the chromophore in the protein.

Photochemical Reaction of Rh7. A transient species, Rh7(580), which was observed in the measurement of absorption spectra, seems to correspond to a photoproduct, Rh7(570), reported by Buchert et al. (1983), because of similarities between their absorption maxima and their decay time constants. However, Rh7(580) directly reverted to the original pigment in the dark, while Rh7(570) was thermally converted to Rh7(640), which reverted to the original pigment. What is the reason for the discrepancies between them? One of the most plausible explanations is the difference between the excitation photon densities of the laser pulses used in the two research groups. The photon density of the excitation pulse used here (60 μJ/1.8 mmϕ) corresponds to 0.2-photon absorption per molecule of Rh7, assuming that all the molecules in the sample uniformly absorb the photon. On the other hand, the photon density used by Buchert et al. was estimated to be 0.45–0.54 photon. Furthermore, the pulse width of their picosecond laser (6 ps) was 3–4 times shorter than that used here (21 ps). Thus their excitation photon density per unit time is about 10 times larger than ours. In fact, the excitation of Rh7 with the pulse, the photon density of which correpsonds to about 2.1 photons per molecule of Rh7, produced Rh7(630), a photoproduct similar to Rh7(640). Therefore, the photoreaction observed by Buchert et al. is probably composed of two photoreactions of Rh7 and Rh7(580) [or Rh7(570)]. Rh7(640), which should be the same photoproduct as Rh7(630), is unlikely to be produced by a two-photon absorption of Rh7 but by a photon absorption of Rh7(580) [or Rh7(570)] because the probability of a two-photon absorption of Rh7 would be very small under this condition.

The discussion described above is in agreement with the fluorescence kinetics of Rh7 (Figure 7), in which the fluorescence was observed only when Rh7 was excited by the intense pulse which produced Rh7(630). Furthermore, the fluorescence intensity was not proportional to the excitation photon density but become higher when the excitation photon density was increased (Figure 7, inset). These facts further support the idea that the generation of Rh7(630) is not due to a single-photon reaction. The reaction scheme is shown in Figure 8b. Rh7(580) is a ground-state species which is generated directly from the excited state of Rh7, and Rh7(630) is generated as the result of a photon absorption of Rh7(580). Fluorescence seems likely to be observable from an excited state of Rh7(580). Rh7(630) may be a ground-state species, since the lifetime of Rh7(630) (Figure 3) is longer than that of the fluorescence (Figure 7).

As already mentioned, the seven-membered ring in Rh7 is more flexible than the five-membered ring in Rh5. Thus the difference in the primary process between Rh5 and Rh7 is due to that in the flexibility of the ring. In other words, the excited

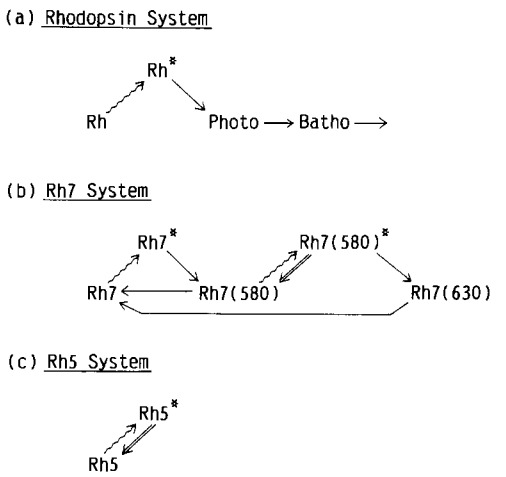

FIGURE 8: Primary photochemical schema in Rh, Rh7, and Rh5. Solid, wavy, and open arrows indicate thermal, photon absorption, and fluorescence processes, respectively.

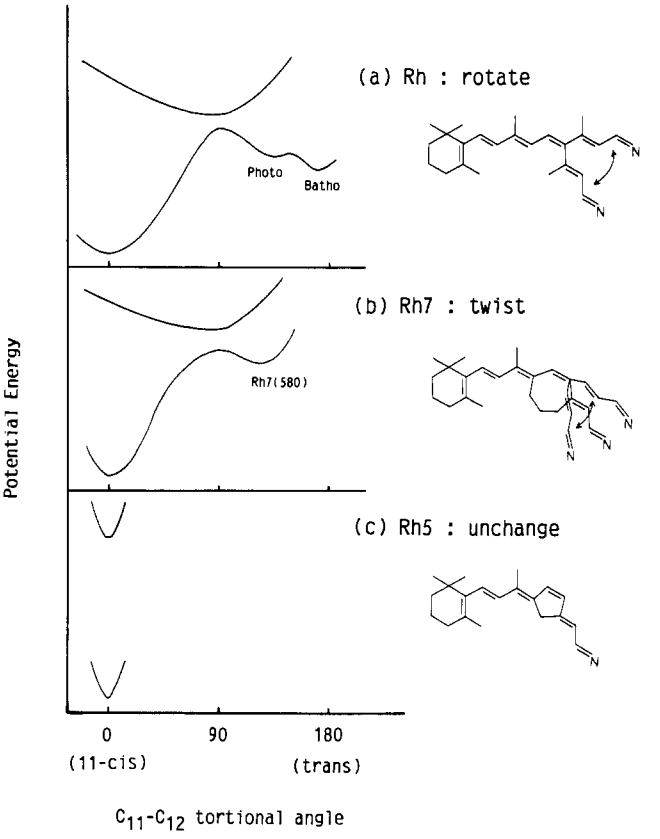

FIGURE 9: Schematic diagrams of the ground- and the excited-state potential surfaces along the C_{11}–C_{12} torsional coordinates of chromophores of Rh (a), Rh7 (b), and Rh5 (c).

state of Rh7 can relax to the original Rh7 through a conformational change of the seven-membered ring moiety of the chromophore. Since Rh7(580) is a ground-state species, the ground-state potential surface of the chromophore of Rh7 has two minima (Figure 9); one may be a relatively relaxed cis configuration [Rh7], and the other may be an highly distorted configuration [Rh7(580)]. What is the structure of the chromophore of Rh7(580), cis or trans form? If Rh7(580) had a cis form, a strong interaction between the chromophore and its neighboring amino acids should exist to stabilize the two kinds of cis form [Rh7 and Rh7(580)] because it would be impossible to search for them in the chromophore itself. Current findings suggest that there is little, if any, protein-induced constraint near the middle portion of the chromophore (Liu & Asato, 1985). Therefore, it is unlikely that a strong

chromophore/protein interaction exists which generates the highly distorted cis configuration.

On the other hand, it was reported that excitation of 1-phenylcyclohexene or *cis*-2-cycloheptenone yielded a transient trans configurational species in the ring moiety (Bonneau et al., 1976; Dauben et al., 1979; Corey et al., 1965; Eaton et al., 1965). Moreover, it was shown that cis–trans isomerization of one double bond occurred in the photochemical reaction of 1,3-cyclooctadiene (Liu, 1967) or 1,3-cycloheptadiene (Inoue et al., 1985). Thus cis–trans isomerization can take place in a seven-membered ring containing four sp^2-hybridized carbons. Although the seven-membered ring in the chromophore of Rh7 is different from these compounds, they suggest that a twisted trans configurtion at the C_{11}–C_{12} double bond of the seven-membered ring can be produced by excitation. Therefore, it is reasonable to assume that the chromophore of Rh7(580) is in a trans-like configuration which does not interact strongly with amino acids near the seven-membered ring (Figure 9b).

There are some differences between Rh7 and PSB7 in the primary process, especially in formation of photoproducts. Upon excitation, Rh7 was converted to Rh7(580), while PSB7 directly relaxed to its original ground state with emission of fluorescence (no ground-state product existed in absorption measurement). Since both ends of the chromophore of Rh7 are fixed by the protein (opsin), it may lead to a stabilized and highly constrained trans configuration of the chromophore. Upon excitation, fluorescence was observed from PSB7 but not from Rh7. This suggests that the protein (opsin) acts as a promoter of conformational and/or configurational changes of the chromophore, especially around the C_{11}–C_{12} double bond.

Primary Dynamics of Rh5, Rh7, and Rh. On excitation of rhodopsin with a picosecond laser pulse, photorhodopsin produces and then decays thermally to bathorhodopsin with a time constant of about 45 ps (Shichida et al., 1984; Kandori et al., 1989). The failure to observe fluorescence upon excitation of Rh suggests that photorhodopsin is a ground-state species. There are remarkable similarities between photorhodopsin and Rh7(580), which are close in difference absorption maximum (585 and 580 nm, respectively) and lifetime (45 and 20–40 ps, respectively). Since in the excited states, both chromophores are forced to rotate around the 11-ene, these similarities indicate that the chromophore of photorhodopsin is in a twisted trans form.

However, there are some differences in nature between photorhodopsin and Rh7(580). Photorhodopsin is thermally converted to bathorhodopsin, while Rh7(580) reverts to the original pigment. Furthermore, Rh7(580) has a broader absorbance than photorhodopsin at longer wavelengths than 600 nm. The quantum yield for formation of Rh7(580) was estimated to be about 0.13 times smaller than that of photorhodopsin, assuming that the extinction coefficient of Rh7(580) is comparable to that of photorhodopsin. Because of the small quantum yield, the C_{11}–C_{12} double bond of the chromophore of Rh7 in the excited state is likely to adopt a conformation that tends to relax to a cis form. Even if it relaxes to a highly twisted trans configuration [Rh7(580)], it may be hard to rotate the C_{11}–C_{12} double bond to form a more relaxed trans configuration, which may be similar in shape to that of bathorhodopsin.

It should be noted that the excited state of Rh5 is considerably different from those of Rh and Rh7 described above. The C_{11}–C_{12} double bond locked with the five-membered ring cannot rotate in the excited state, so that the Franck–Condon state and the excited equilibrium state are similar in structure of the chromophore to each other. Probably this leads to a long-lived excited singlet state of Rh5. On the basis of these discussions, schematic potential surfaces of Rh, Rh7 and Rh5 systems are shown in Figure 9.

Finally, the primary dynamics of Rh5, Rh7, and Rh systems will be summarized. In Rh, the energy of an absorbed photon would be concentrated into the C_{11}–C_{12} double bond, and used for rotation. If the rotation of this bond is definitely prohibited, as it is in Rh5, the molecule in its excited state has to relax so as to dissipate the energy of a photon by internal conversion. The internal conversion processes would be harder in the chromophore binding site of opsin than in solvent. On the other hand, the lifetime of the excited state of Rh7 was shorter than that of PSB7. This fact suggests that the opsin accelerates the rotation of the C_{11}–C_{12} double bond. Therefore, a large quantum yield for isomerization of the chromophore of Rh (0.67) may be caused by fixation at both ends of the chromophore by the protein, resulting in an increase of the efficiency of the energy concentration into rotation at the center of the chromophore and a decrease of the efficiency of the internal conversion processes.

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Registry No. Ret5, 83631-39-4; Ret7, 79270-63-6; PSB5, 121125-42-6; PSB7, 121125-43-7.

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Chromophore States in Allophycocyanin and Phycocyanin. A Resonance Raman Study†

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ABSTRACT: UV-excited, low-temperature resonance Raman spectra were recorded from the trimer and the pH-induced monomer and denatured forms of allophycocyanin from *Synechococcus* 6301 (*Anacystis nidulans*). Monomerization mostly results in changes in relative intensities of Raman bands of the chromophore(s), a likely consequence of changes in their excited electronic states associated with the depletion of the 652-nm excitonic band. Frequency shifts of modes involving motions of the pyrrolic nitrogens indicate that monomerization also results in environmental changes around the chromophores, possibly involving H-bonding of their nitrogens. No such phenomena were observed during monomerization of C-phycocyanin. These events, however, do not include any sizable change in the native, fully extended conformations of any of the two chromophores, as manifested by the absence of any sizable shift or broadening of the 1642-cm^{-1} marker band. Upon monomerization, resonance Raman spectra of allophycocyanin become very similar to those of monomeric or trimeric C-phycocyanin, indicating that the conformations and environmental interactions of the chromophores must be very close to each other in the two proteins. Similarly, in the denatured states, the chromophores of both proteins adopt very similar, cyclohelical conformations, close to those of free chromophores in vitro. Yet the denaturation processes follow significantly different routes for allophycocyanin and C-phycocyanin. In particular, allophycocyanin monomers appear more sensitive to pH-induced denaturation than C-phycocyanin monomers.

Phycobilisomes, the light-harvesting complexes of cyanobacteria and red algae (Gantt, 1981), involve the assemblies of biliproteins. The prosthetic groups of these proteins are covalently bound open-chain tetrapyrrole molecules named phycocyanobilins. The phycobilisomes of *Synechococcus* 6301 (*Anacystis nidulans*) contain two types of biliproteins, C-phycocyanin (CPC)[1] and allophycocyanin (APC), the native forms of which are oligomeric. The monomers are equimolar associations of two nonidentical polypeptide chains, α and β. The α subunit carries one covalently bound phycocyanobilin molecule in both proteins. In APC the β subunit also binds one phycocyanobilin, while the β subunit of CPC binds two [for a review see Glazer (1984)]. Both CPC (MacColl et al., 1971) and APC can be monomerized. The monomers are very similar in their absorption and fluorescence spectra (MacColl et al., 1980). Therefore, it is tempting to assume that the chromophore structures as well as the protein environments are also rather similar in CPC and APC monomers (Murakami et al., 1981).

MacColl et al. (1980) discussed the electronic absorption spectra of APC and CPC. They showed that the visible absorption spectrum of native trimeric phycocyanin only differs by a red shift from that of the monomers. In contrast, trimeric

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[1] Abbreviations: APC, allophycocyanin; CPC, C-phycocyanin.

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