Real-Time Detection of 60-fs Isomerization in a Rhodopsin Analog Containing Eight-Membered-Ring Retinal

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Abstract: Femtosecond fluorescence spectroscopy has been used to investigate the excited-state dynamics of a rhodopsin analog having retinal modified by a flexible eight-membered ring. The fluorescence lifetime was determined to be 60 fs throughout the observed wavelengths. Combined with the previous transient absorption results (Mizukami, T.; Kandori, H.; Shichida, Y.; Chen, A.-H.; Derguini, F.; Caldwell, C. G.; Bigge, C. F.; Nakanishi, K.; Yoshizawa, T. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4072-4076), the ultrafast process was interpreted in terms of rapid deformation around the C11−C12 double bond, i.e., isomerization, occurring as fast as vibrational motions of the chromophore. The ultrafast reaction suggests that the isomerization occurs in a coherent manner. On the basis of the present results for the modified rhodopsin, the primary processes in the native rhodopsin are discussed.

Introduction

Rhodopsin, a photoreceptor protein present in our eyes, is an excellent molecular switch to convert a light signal to the electrical response of the photoreceptor cells.[1] Since the ultrafast isomerization of the rhodopsin chromophore is one of the essential properties of rhodopsin to capture light signals so efficiently, elucidation of the isomerization mechanism has been one of the central issues in vision as well as ultrafast spectroscopy.[2] We have studied the primary isomerization mechanism by applying picosecond spectroscopy to rhodopsin analogs possessing 11-cis-locked-ring retinals as their chromophores (Figure 1).[3,4] In these rhodopsin analogs, 11-cis-locked retinals with five-, seven-, and eight-membered rings (Ret5, Ret7, and Ret8, respectively) are incorporated into bovine opsin, so that the formed pigments become unbleachable. In spite of no bleaching of these pigments, interestingly, picosecond transient absorption spectroscopy provided different photophysical and photochemical properties among them.

In the case of five-membered rhodopsin (Rh5), only a long-lived excited state (τ = 85 ps) and no product formation were observed (Figure 1D), providing direct evidence that cis−trans isomerization is the primary event in vision. Excitation of seven-membered rhodopsin (Rh7), on the other hand, yielded an appearance of the ground-state photoproduct that had a spectrum similar to that of photorhodopsin, the primary intermediate of rhodopsin (Figure 1C).[5] These different results

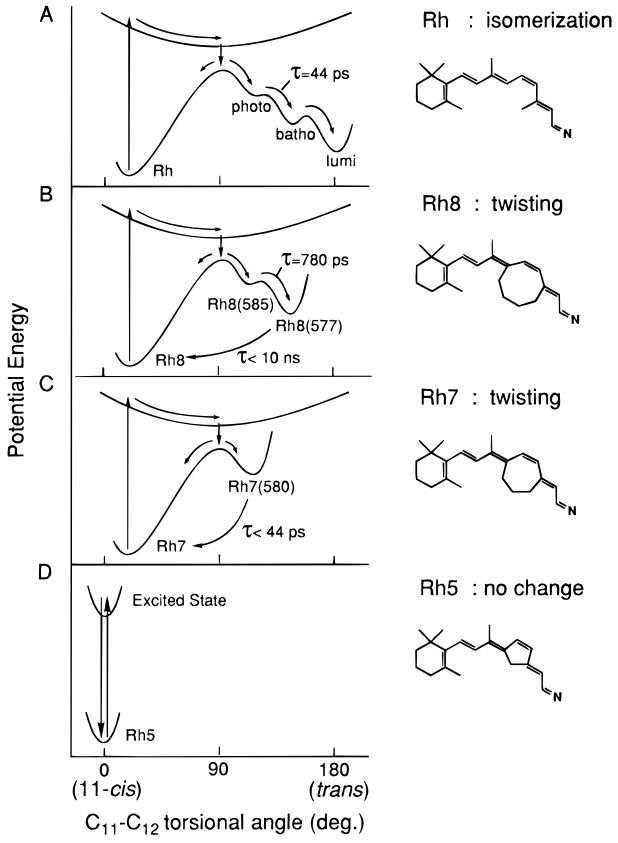

Figure 1. Schematical drawing of ground- and excited-state potential surfaces along the 11-ene torsional coordinates of the chromophore of rhodopsin (A), Rh8 (B), Rh7 (C), and Rh5 (D).

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were interpreted in terms of the rotational flexibility along the C11−C12 double bond.[3] This hypothesis turned out to be more reliable with the recent results on eight-membered rhodopsin (Rh8), which possesses a more flexible ring.[4] Upon excitation of Rh8 with a 21-ps pulse, two photoproducts were observed, not only photorhodopsin-like but also bathorhodopsin-like products (Figure 1B). Thus, the picosecond absorption studies directly elucidated the correlation between the primary processes of rhodopsin and the flexibility of the C11−C12 double bond of the chromophore, and we eventually inferred the respective potential surfaces as shown in Figure 1.[4]

Among the spectroscopic properties of 11-cis-locked rhodopsins, those of Rh8 are particularly noteworthy. Rh8 has λ_{max} at 500 nm, which is closer to that of the native rhodopsin (λ_{max} = 498 nm) than those of Rh7 (λ_{max} = 490 nm) and Rh5 (λ_{max} = 495 nm). In addition to the formation of photo- and batho-like products, the dependence of an absorbance increase on the photon density is similar in Rh8 to that in rhodopsin under the same excitation conditions, indicating that the quantum yield of the product formation in Rh8 is very similar to that in rhodopsin.[4] This is contrary to the case of Rh7, whose quantum yield is 5 times less than that of rhodopsin.[3] These facts indicate that the primary photochemical nature of Rh8 is close to that of the native rhodopsin. Therefore, in this paper, we extended our research on the isomerization mechanism of rhodopsin by investigating the excited-state dynamics of Rh8.

So far, the primary processes of rhodopsin have been investigated predominantly by (transient) absorption spectroscopy,[2] which can observe the product absorption directly. Recent progress in generating a femtosecond pulse enabled one to observe the excited-state dynamics of rhodopsin, followed by the formation of primary intermediates.[6−8] To probe the excited-state dynamics of rhodopsins, however, absorption spectroscopy may not be advantageous, since the other spectral features, such as ground-state depletion and product absorption, are possibly superimposed on the excited-state spectral features (absorption and stimulated emission) in the obtained data.[9] Each spectral feature may even vary in the femtosecond regime, which provides further difficulty in analyzing the data. On the other hand, the fluorescence spectroscopy focuses only on the excited-state processes, so that the excited-state dynamics is observed more directly.[10]

In order to investigate the excited-state dynamics of Rh8, we have constructed a femtosecond fluorescence spectrometer based upon the "up-conversion" technique, by which an extraordinarily weak fluorescence with femtosecond resolution was detected.[11] As described above, the properties of the excited state of Rh8 are similar to those of the native rhodopsin, indicating that Rh8 is a suitable model pigment to elucidate the excited-state dynamics of rhodopsin.[4] Another advantage is that the primary photorhodopsin-like product does not bleach but reverts to the original pigment within several nanoseconds after converting to the next bathorhodopsin-like intermediate (Figure 1B). Thus, the unbleachable properties of Rh8 with the rapid reversion (<10 ns) are highly advantageous for femtosecond experiments using high-repetition laser pulses.

Experimental Section

Preparation of Rh8 and Its Spectroscopic Characteristics. The Rh8 sample for femtosecond experiments was prepared from bovine opsin and Ret8 as described previously.[4,12] Incubation of bovine opsin with 11-cis-locked eight-membered retinal (Ret8) formed two pigments absorbing at 425 nm (P425) and 500 nm (P500). P425, however, is an artificial pigment because it formed from thermally denatured opsin or other proteins and Ret8. P500 was isolated and purified from the reaction mixture by means of concanavalin A−Sepharose column chromatography. Absorbance of the Rh8 sample at 500 nm for the present experiments was 0.53 per 1-mm path length.

Femtosecond Up-Conversion Apparatus. A femtosecond up-conversion apparatus was used to measure the fluorescence from Rh8.[11] The light source was a mode-locked Ti/sapphire laser, whose central wavelength, pulse width, and repetition rate were 888 nm, 100 fs, and 82 MHz, respectively. The second harmonic (444 nm) was used for excitation of Rh8. The Rh8 sample of 100-μL volume was put in a 1-mm-path-length cell and stirred with an electric brush during the experiments. No absorption change of the sample was observed after the experiments. The excitation energy and the diameter of the laser beam were 70 mW (0.8 nJ/pulse) and ∼100 μm, respectively, so that the photon density was estimated at 2.5×10^{13} photons/cm^2. This corresponds to 0.2% excitation of Rh8 molecules in the sample. Fluorescence from the Rh8 sample was collected in the same direction as the excitation beam. Both the fluorescence and the fundamental beams, the latter of which was passed in advance through an optical delay line, were focused onto a phase-matched 0.2-mm BBO crystal, which led the formation of ultraviolet sum-frequency light. The UV light was detected with a photomultiplier coupled to a single-photon counting apparatus. Each data point in the figures is the average of three independent experiments with an integration time of 1 s. The present instrumental response function was obtained by measuring the cross-correlation between the excitation and gate pulses, which had a Gaussian shape, and the half-width was 180 fs.

Results and Discussion

Excited-State Dynamics of Rh8. Before applying femtosecond fluorescence experiments, we attempted to measure a steady fluorescence spectrum of Rh8 with a conventional fluorescence spectrophotometer. The emission from Rh8 was, however, too weak to be detected. The comparative experiments using erythrosin as a standard molecule[13] indicated the fluorescence quantum yield of Rh8 (ϕ_f) to be lower than 5×10^{-5}.

The Rh8 sample was excited with a 444 nm femtosecond pulse (a second harmonic of the Ti/sapphire laser), and a time-resolved fluorescence was observed. Typical fluorescence decay kinetics of Rh8 are shown in Figure 2A. The fluorescence signal at 578 nm decays very rapidly, which is close to the instrumental response (broken line in Figure 2A). A small fluorescence signal (∼10% of the peak) is also observed between 400 and 600 fs, which persists even at 20 ps. This component is not coupled to the processes forming the photorhodopsin-like product, because the product forms within 20 ps.[4] The slow component may originate from (i) an excited state of Rh8 that is not coupled to the product formation such as a "forbidden"

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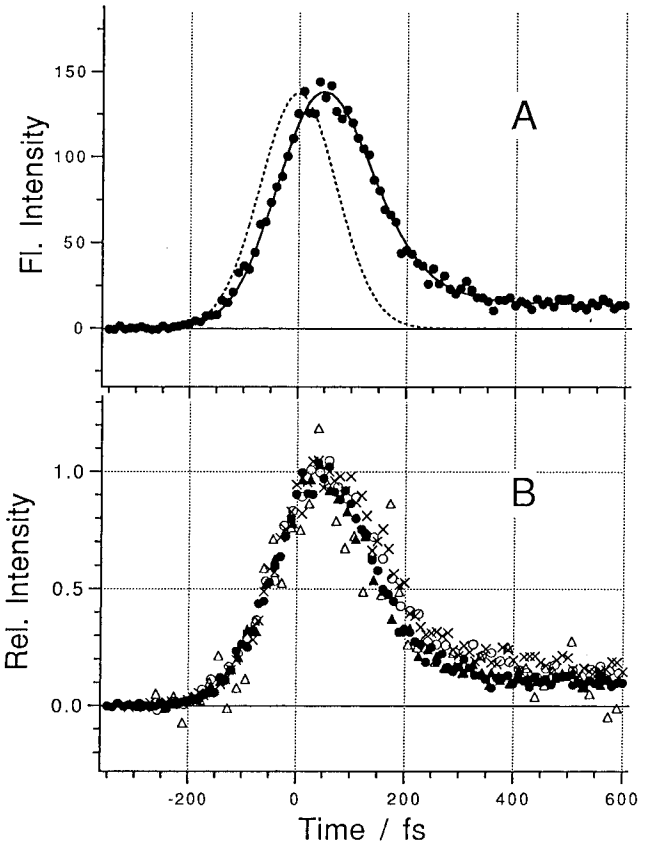

Figure 2. (A) A typical fluorescence decay of Rh8 recorded at 578 nm. The solid line is the best fitting curve, which was obtained by convolution with the instrumental response function (broken line) and a decay of 60-fs lifetime. (B) Peak normalized fluorescence decay curves at five wavelengths. The actual fluorescence intensity was 80, 130, 130, 70, and 12 counts at the peaks for 551 (×), 578 (●), 605 (▲), 634 (○), and 664 (△) nm, respectively, where transmittance of the filter, the photomultiplier sensitivity, and self-absorption by the sample are not calibrated.

^{1}Ag*-like excited state,[14] (ii) a higher electronically ^{1}Bu*-like state such as a β-band, or (iii) an excited state of any impurity, as is observed in the experiments of native rhodopsin.[13,15] The lifetime of the fast component was estimated to be 60 ± 10 fs by the deconvolution procedure (Figure 2A).

Since the decay time constant of the fluorescence is extremely short, we first implied that the decay might be due to the intramolecular relaxation originating from the excess energy provided by the excitation pulse (444 nm). If this is the case, the time-dependent red shift of the emission spectrum (called the "dynamic Stokes shift" [16]) should be observed. Namely, the time-resolved fluorescence at shorter wavelength decays rapidly, while that at longer wavelength shows rise components. Therefore, we next investigated the wavelength dependence of the fluorescence kinetics by changing the probing wavelength. The normalized fluorescence kinetics at five wavelengths from 551 to 664 nm (Figure 2B) showed the same decay (60 ± 20 fs), indicating that the dynamic Stokes shift was not observed in the relaxation processes. Thus, the origin of the extremely fast decay of the excited state is likely due to the conformational change of the chromophore probably through a specific mode for the cis−trans isomerization.

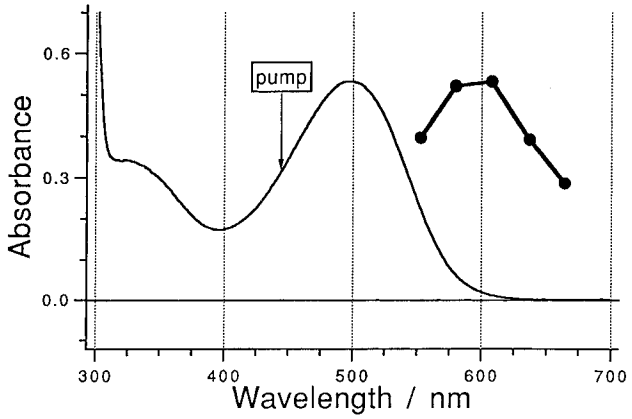

Figure 3. Absorption and emission spectra of a rhodopsin analog having 11-cis-locked eight-membered-ring retinal (Rh8). The absorbance of the final Rh8 sample at 500 nm for the present experiments was 0.53 per 1-mm path length. The present emission spectrum (closed circles) was estimated from the fluorescence intensity of the fast kinetic component (Figure 2B) after correcting the wavelength dependence of the filter, monochromator, detector, and sample absorption. It should be noted that the fluorescence spectrum is similar in shape to that observed from the native rhodopsin.

The fluorescence quantum yield of Rh8 was estimated to be on the order of 10^{-6} ($\phi_f \approx 6 \times 10^{-6}$) using the lifetime (60 fs) and the natural radiative lifetime (∼10 ns) of Rh8.[4] Absence of the dynamic Stokes shift suggests that the isomerization is a faster event than the intramolecular relaxations. These experimental observations meet well with a barrierless potential surface along the C11−C12 rotational coordinate (Figure 1), which has been widely accepted for the native rhodopsin on the basis of the temperature- and excitation wavelength-independent photoisomerization.[17] Furthermore, the 60-fs decay (∼560 cm^{-1} in the frequency domain) is comparable to the periods of torsional deformations of the chromophore.[18] Therefore, the fluorescence lifetime is probably faster than the vibrational dephasing time, suggesting that the isomerization is a coherent process.

The excited-state dynamics of Rh8 was wavelength-independent, whereas the intensity of the fluorescence signal varied with wavelength (see the legend of Figure 2B). We then estimated the fluorescence emission spectrum of Rh8 by using the intensity of the 60-fs component (Figure 3). The emission maximum is located at about 600 nm, in good agreement with that obtained for the native rhodopsin (bovine, 600 nm; squid, 620 nm).[13] It should be noted that the emission spectrum of Rh8 displays a considerable Stokes shift (Figure 3), indicating that the detected fluorescence does not originate from the Franck−Condon state. In other words, there is a relaxation process from the Frank-Condon state to the fluorescence state within the present time resolution (≪60 fs).

By combining the results of picosecond transient absorption and femtosecond fluorescence measurements, the detailed description of the excited-state isomerization dynamics in Rh8 is now opened to be resolved. The Franck−Condon excited state, which is described by reference to the ground state geometry from which the electronic excitation occurs, has an extremely short lifetime. Therefore, the energy of a photon absorbed by the chromophore is quickly dissipated or distributed among high-frequency modes of the vibration. Only high-frequency vibrations, such as C═C stretching modes, could

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participate in the relaxation from the Franck−Condon state in view of the time scale (≪60 fs). It is difficult to characterize the fluorescence property from the Franck−Condon excited state because of its short lifetime, though it should have a large fluorescence quantum yield. The observed fluorescence mainly originates from the excited state other than the Frank−Condon state, which we define as the "FL state". The wavepacket on the excited-state potential surface rapidly moves from the Franck−Condon state along the trajectory to the FL state prior to complete internal conversion. The FL state probably has a chromophore whose geometry is different from that in the ground state. The absence of a dynamic Stokes shift in the fluorescence decay suggests that the fluorescence state may decay through the specific vibrational modes of the chromophore, including the torsional mode of the C11−C12 double bond. With the lifetime of 60 fs, the FL state further relaxes, and probably reaches a nonfluorescence state, from which the primary ground state species Rh8(585) is formed. The formation time of Rh8(585) is shorter than 21 ps as revealed by picosecond absorption spectroscopy.[4] Although the present experiments clearly show the excited-state dynamics of Rh8, the direct evidence of the structure of the chromophore in these excited states would be given by applying time-resolved vibrational spectroscopy. In addition, the complete description of the process of product formation in Rh8 will be resolved by ultrafast absorption measurements.

Implications of the Excited-State Dynamics in Rhodopsin. Recent time-resolved absorption spectroscopy clearly shows the ultrafast formation of the primary intermediate of rhodopsin, photorhodopsin, from an excited state of rhodopsin,[6] and the occurrence of a coherent isomerization by probing the wavelength dependence of the oscillatory signal in the femtosecond time regime.[19] However, the excited-state dynamics of rhodopsin was not argued in detail in these papers, though the time resolution reached several tens of femtoseconds. The reason might be mainly the spectral contamination of the other spectral features, such as ground-state depletion, product absorption, and the excited-state spectral features (absorption and stimulated emission). Thus, it is worthwhile to imply the excited-state dynamics of rhodopsin from the experimental results obtained from Rh8.

In addition to the similar quantum yield for the formation of the primary intermediates between rhodopsin and Rh8, there is other evidence that supports the similar excited-state potential surface between these pigments. According to the analysis of the time-correlated function of the wavepacket propagation in the excited state of rhodopsin from a Fourier transform of optical absorption spectra (FTOA),[20] the wavepacket on the excited-state potential surface of rhodopsin rapidly moves from the Franck−Condon state within 30 fs, partially returns after about 50 fs, and finally moves away from the Franck−Condon state. These implications are consistent with those observed for Rh8. Thus, it is reasonable to speculate that the excited-state potential surface of rhodopsin is similar to that of Rh8.

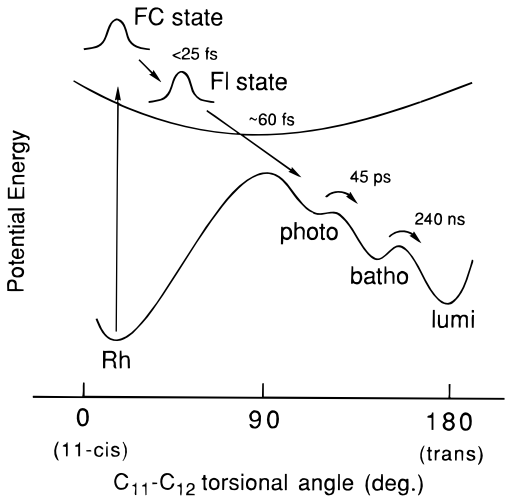

Figure 4. Schematical drawing of the potential surfaces along the 11-ene torsional coordinate of rhodopsin.

On the basis of the present findings and above arguments, the potential surface of rhodopsin is schematically drawn in Figure 4. In rhodopsin, as well as Rh8, the Franck−Condon state has an extremely short lifetime, and therefore the photon energy absorbed by the chromophore is quickly dissipated or distributed among high-frequency modes of the vibration. There should be another state like the FL state in its excited state in analogy to Rh8, from which the transition to photorhodopsin occurs. However, direct proof should be provided by the measurements of the native rhodopsin, which is our future focus.

Visual isomerization is one of the fastest chemical reactions observed so far, and is achieved in the specific protein environment of rhodopsin. Thus, animals have acquired the specific protein to obtain high photosensitivity of vision. The present study opens the question of how the protein environment can control the ultrafast reaction of the chromophore, which may be tested by ultrafast spectroscopy with the aid of rhodopsin mutants.

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