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Review

Sub-5-fs Real-time Spectroscopy of *Transition States* in Bacteriorhodopsin During Retinal Isomerization†

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ABSTRACT

By using a sub-5-fs visible laser pulse, we have made the first observation of the vibrational spectra of the transition state during *trans-cis* isomerization in the retinal chromophore of bacteriorhodopsin (bR_{S68}). No instant isomerization of the retinal occurs in spite of electron promotion from the bonding π-orbital to the anti-bonding π^*-orbital. The difference between the in-plane and out-of-plane vibrational frequencies (about 1150–1250 and 900–1000 cm^{-1}, respectively) is reduced during the first time period. The vibrational spectra after this period became very broad and weak and are ascribed to a "*silent state*." The silent state lasts for 700–900 fs until the chromophore isomerizes to the *cis*-$C_{13}=C_{14}$ conformation. The frequency of the C = C stretching mode was modulated by the torsion mode of the $C_{13}=C_{14}$ double bond with a period of 200 fs. The modulation was clearly observed for four to five periods. Using the empirical equation for the relation between bond length and stretching frequency, we determined the transitional C = C bond length with about 0.01 Å accuracy during the torsion motion around the double bond with 1-fs time resolution.

INTRODUCTION

Developments in short pulse lasers allow ultrafast chemical reaction dynamics and femtochemistry to be studied (1,2). The primary photochemistry for the retinal chromophore of bacteriorhodopsin (bR_{568}) has been studied in the context of its *cis–trans* isomerization (3).

Zewail *et al.* (4) started "transition state spectroscopy" and succeeded in observing the dynamical motion of a wave packet along a reaction coordinate and the crossing between two potential surfaces described by the Landau–Zener (LZ) tunneling mechanism (5,6) and more recently in terms of conical intersection of the surfaces (7).

Bacteriorhodopsin, bR_{568}, undergoes the following photochemical cycle:

$$bR_{568} \xrightarrow{h\nu} H(\tau < 50\ \text{fs}) \rightarrow I_{460}(50\ \text{fs} < \tau < 100\ \text{fs}) \rightarrow J_{625}(100\ \text{fs} < \tau < \approx 1\ \text{ps}) \rightarrow K_{610} \rightarrow L_{543} \rightarrow M_{412} \rightarrow N_{560} \rightarrow O_{640} \rightarrow bR_{568}$$

Here, H denotes the Franck–Condon excited state of bR_{568} with its all-*trans* retinal Schiff base. The subscripts represent the absorption maxima of the species. Several vibrational spectroscopic studies using ultrashort pulses (8–12) have shown that H and I_{460} are excited-state species with the chromophore still in its all-*trans* conformation and that K_{610} is a ground-state species with the chromophore already converted to the 13-*cis* conformation. However, the primary molecular processes between the H and K_{610} states have been controversial.

Two models have been proposed for the photoisomerization process, the two-state (13–15) model and the three-state model (16–19). However, recent data from pump-probe experiments on bR_{568} shows that there is no spectral change in the stimulated emission spectrum during the period of the excited state lifetime (30 fs–1 ps delay time region) (16–18). Also spontaneous fluorescence lifetime measurements (19) show that there is almost no dynamic Stokes shift during the period of the excited state lifetime. If bR follows the two-state model, the K state should appear during the period of the excited state lifetime, which causes the stimulated emission spectrum to be modified and the dynamic Stokes shift. So these experimental results cannot be explained by the two-state model but instead support the three-state model (20).

Pump-probe experiments for bR analogs containing synthetic $C_{13}=C_{14}$ locked chromophores with *cis* and *trans* configurations (21,22) showed the appearance of an I_{460}-like

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species within 30 fs. The close resemblance in the initial transient spectral evolution of both native and the artificial pigments indicates that the ultrafast process from H to I_{460} does not involve $C_{13}=C_{14}$ torsion, so I_{460} is still in an all-*trans* conformation. Furthermore, recent papers have proposed that the first event is not the *trans-cis* isomerization but a skeletal stretching from the H state (12,21–23). Recent *ab initio* calculations of photoisomerization dynamics have suggested that skeletal deformation takes place about 50 fs before $C_{13}=C_{14}$ torsion takes place (24,25).

In this work, we have measured the real-time absorption change spectra and observed the modulations in the Fourier power and instantaneous frequency of the molecular vibrations. The information shows what is going on during the photochemical transformation between bR_{568} and K_{610}.

MATERIALS AND METHODS

In the present work, pump and probe pulses were generated by a noncollinear optical parametric amplifier (NOPA) seeded by a white-light continuum with a 5-fs pulse compressor system (2,26,27). The pump source for the NOPA is a regenerative amplifier CPA1000 (MXR Clark), whose pulse duration, central wavelength and repetition rate are 120 fs, 790 nm, 1 kHz, respectively. Both of the spectra of pump and probe pulses are nearly the same and cover a spectral range from 500 to 710 nm with a nearly constant phase. All measurements were performed at room temperature (21°C).

RESULTS AND DISCUSSION

In order to help sort out the different models discussed above, we measured the time-resolved absorbance changes (ΔA), which we call the real-time signal, of bR_{568} using sub-5-fs pulses as both pump and probe pulses. Figure 1 shows the 2D absorbance change probed at delay times between −100 and 800 fs. The bR was excited with sub-5-fs pulses from the NOPA. Both of the Fourier power spectra calculated in the spectral ranges of induced absorption and bleaching show a 1500–1550 cm^{-1} band. Resonance Raman studies of bR were performed by Mathies *et al.* (10,32). In their study, the modes in the 1500–1550 cm^{-1} region was assigned to the C=C stretching modes. Upon the photoisomerization of retinal, the carbon double bond between C_{13} and C_{14} is deformed. Therefore the modes in the 1500–1550 cm^{-1} region are mainly dominated by the C=C bond stretching modes especially localized in the $C_{13}=C_{14}$ double bond where photoisomerization takes place.

Real-time signals of bR_{568} at several wavelengths revealed that the measured ΔA is modulated by vibrational modes that are coupled to stimulated emission or excited-state absorption and have low enough frequencies so that the excitation pulse excites multiple levels of the mode (*i.e.* creates a wave packet) (28,29). The Fourier transformation of ΔA around an arbitrary delay time t_d gives the amplitudes and instantaneous frequencies of the molecular vibrations at t_d. We calculated spectrograms for all of the real-time signals probed in broadband spectrum of the probe pulse (30). To calculate the spectrograms, we have used windows whose widths were 4 nm and 200 fs in the time and probe wavelength regions, respectively. And these spectrograms that show the instantaneous frequencies are modulated with a period of about 200 fs. Figure 2 shows the spectrograms of the real-time traces probed at 585 and 640 nm corresponding to the bleaching and induced absorption spectral ranges, respectively. In (a) and (b), the intensities of the in-plane C=C–H bending and C–C stretching modes (1150–1250 cm^{-1}) are partially recovered at 700–900 fs. In (c) and (d), both of them are modulated both in terms of instantaneous frequency and intensity. It was found that Fourier amplitude and instantaneous frequency of the C=C stretching mode are modified by 18% and 0.9%, respectively, from the mean amplitude and instantaneous frequency.

Our Fourier spectra results were compared with the Raman frequencies (10,31,32) previously measured for the 630 nm probe data. As was observed in the previous Raman measurements, we expected to observe three signals, which consist of the C=C and C=N stretching modes (1500–1650 cm^{-1}) and the in-plane C=C–H bending mode coupled with C–C stretching modes (1150–1250 cm^{-1}), and hydrogen-out-of-plane (HOOP) mode (800–1050 cm^{-1}). All of the signals for the vibrational modes were observed, but the instantaneous frequencies of the vibrational modes were modulated with the delay time. We conclude that the temporal shifts in frequency are due to wave-packet motion of the excited-state rather than ground-state for the following three reasons (1). The spectrogram calculated for the real-time signal at 640 nm due to excited-state absorption has the same features as those at other wavelengths (2). The pulse width (sub-5 fs) is much shorter than the observed molecular vibration frequencies and is too short to enhance the molecular vibration in the ground electronic state (3,28,29). The decay of the molecular vibration amplitude is not longer than that of ΔA. A recent study in our group (data not shown, work submitted), revealed that the contributions from the wave packets in the ground-state, the excited-state and the intermediate all exist in differing amounts depending on the probe wavelength.

A key feature of the spectrogram is the frequency of the ethylene-like symmetric C=C stretching modes in the 1500–1550 cm^{-1} region reflecting π-bond order. Interestingly, the peak frequency is modulated between 1500 and 1550 cm^{-1}. A similar modulation of instantaneous frequencies has previously been observed for polydiacetylene (PDA) using the same sub-5-fs visible pulse (27,33). In PDA, the frequencies of C=C and C–C stretching are modulated at the vibrational period of the C=C–C bending mode (145 fs) for about 2 ps. The modulation frequency in bR_{568} was found to be 160 cm^{-1} from the Fourier transform calculation of the Fourier power spectrum, which corresponds to the 200-fs oscillation period of twisting around the C=C bond. The reason why we assigned the 200-fs oscillation to the twisting around the C=C bond is as follows. According to Eyring *et al.* (31), the period of CCCC torsion was ~200 cm^{-1}. The CCCC torsion twists the carbon double bond in the middle and changes the bond length of the carbon double bond. It modulates the frequency of C=C stretching mode. Therefore we conclude that the modulation period of 160 cm^{-1}, which we have observed on the C=C stretching mode was caused by the twisting motion around the C=C bond during the CCCC torsion process. This is the first real-time observation of the frequency modulation of C=C stretching associated with the torsion motion relevant to the *trans–cis* isomerization. The frequency modulation induces the generation of relevant side bands but resonance Raman

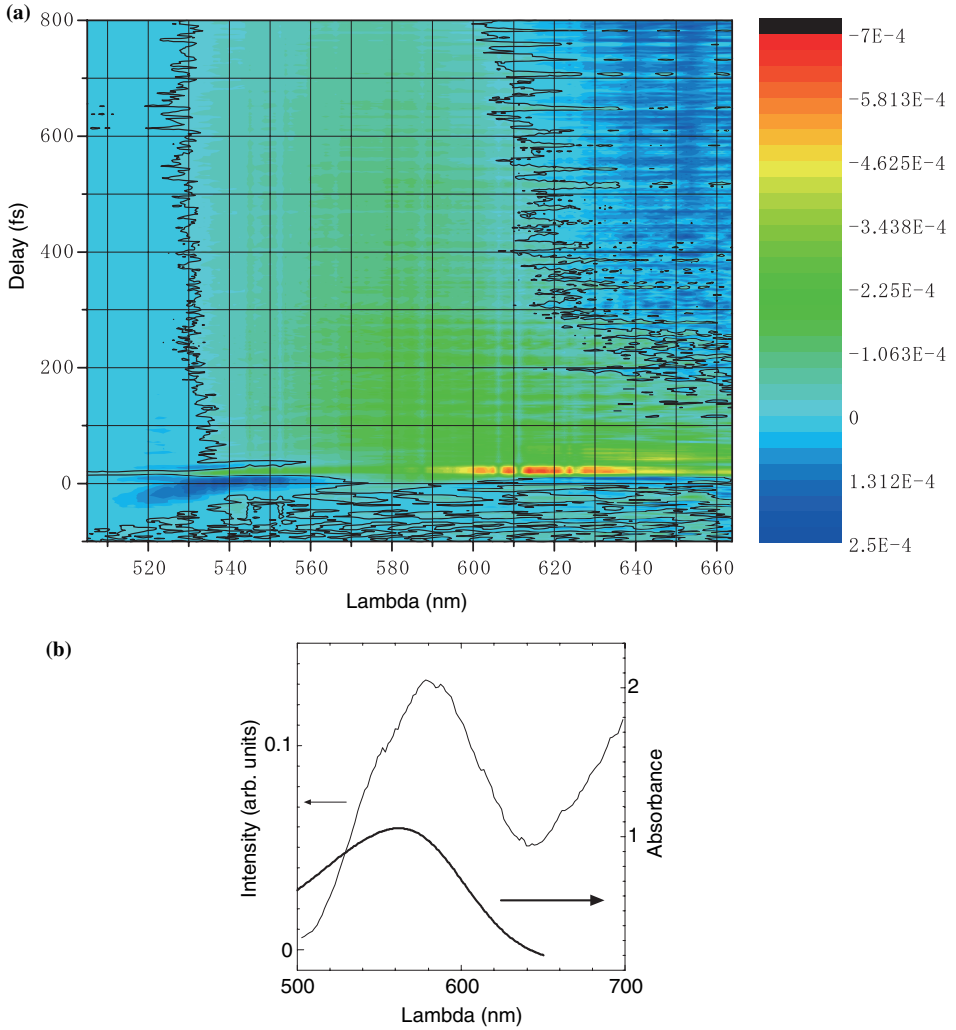

Figure 1. (a) Real-time absorption change trace of bacteriorhodopsin (bR). (b) Laser spectrum of the probe pulses (thin lines) and absorption spectrum of the bR sample.

spectroscopy cannot discriminate the bands from accidental modes existing nearby. The fast dumping causes spectral broadening, whereas resonance Raman spectroscopy cannot distinguish between homogeneous and inhomogeneous broadening. The present method of observing the time-dependent molecular vibration can distinguish the two by detecting the recurrence in the case of inhomogeneous broadening.

Hereafter, we examine the contribution of the 200-fs torsion motion to the coupling of other vibrational modes by describing four events over different time scales. The first event occurs within 50–100 fs. The wave packet reaches a potential region in which the potential is flat along the CCCC torsion coordinate (I_{460} with a B_u-like electronic configuration) from H. Periodic out-of-plane distortion also begins. An intense C=N stretching band from the protonated Schiff base ($C_{15}=NH^+$,1620–1630 cm^{-1}) appears within 50 fs. Both the intensity and frequency of the band dramatically decrease within 100 fs. The fast frequency lowering indicates the effect of the torsion motion. The in-plane C=C–H bending modes coupled with C–C stretching modes appear in the 1150–1300 cm^{-1} region. The region is called the fingerprint region, because it is very sensitive to chromophore conformation. The skeletal vibrations around 1250 cm^{-1} are activated in the first 100 fs as predicted theoretically (24,25). The HOOP modes appear just after the excitation in the 800–1050 cm^{-1} region. The intensity of the HOOP modes is sensitive to the tilting angle around the C=C bond. It is expected that the HOOP intensity at first increases with the torsion angle and then it decreases or even disappears as the angle is close to 90°.

The second event is seen in the 100–200 fs region. The band around 1250 cm^{-1} splits into two peaks after 100 fs. The frequency of one peak gradually increases, reaching 1520–1540 cm^{-1} at about 150 fs, while the frequency of the other peak gradually decreases and it becomes as low as 1130 cm^{-1} at 200 fs after excitation. The temporal shift of the frequency of the latter mode (C=C–H in-plane bending) is correlated with that of the HOOP band. These frequencies are modulated with 200-fs period of the CCCC torsion. Noticeably, the peak positions of the HOOP mode and the in-plane C=C–H bending modes are well separated in the <100 fs region, but not in the 150–200 fs region. These mode frequencies approach each other and merge into a single peak, as there is neither an in-plane nor an out-of-plane mode in a distorted configuration. The two modes reappear in the slightly longer than 200-fs region and planarity is recovered. This

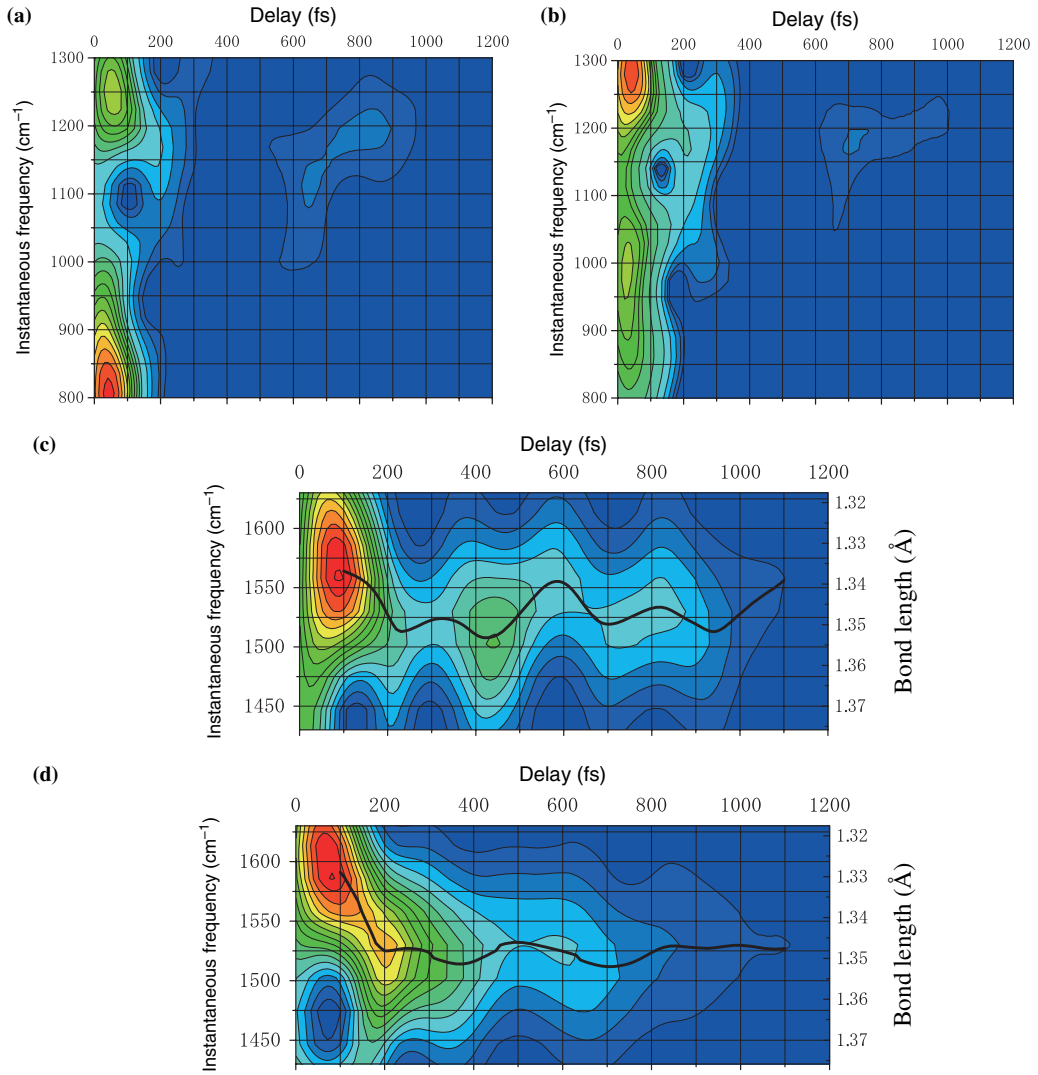

Figure 2. The spectrograms of the real-time traces probed at (a and c) 585 nm and (b and d) 640 nm corresponding to the bleaching and induced absorption spectral ranges, respectively. (a) and (b) are the spectrogram of the 800–1300 cm^{-1} range. (c) and (d) are the spectrogram of the 1425–1630 cm^{-1} range.

suggests that the *trans* → 13-*cis* isomerization occurred within < 200 fs according to the two-state model. However, the product K_{610} has not yet been formed. For the t_d > 200 fs region, the intensities of the C=C–H bending and HOOP modes become very weak at all the probe photon energies studied (610–680 nm, data not shown). Therefore, the coincident increase and decrease of the frequencies of the HOOP and in-plane C=C–H bending modes strongly indicate that the torsion modifies both the out-of-plane and in-plane bending-mode frequencies with the period of 200 fs.

The third event is the formation of the transition state in the 300–600 fs region. C=N stretching, C=C–H bending and HOOP modes are also silent in this region. The weak intensity is partly due to the broad distribution of molecular conformations covered by the wave packet. Energy randomization occurs during the fast wave-packet motion along the potential curves of the excited B_u-like and A_g-like states and during the LZ tunneling and/or conical intersection in between B_u and A_g. However, the isomerization in the $C_{13}=C_{14}$ bond has not occurred in this stage. The skeletal change precedes the isomerization. We call the molecular state in the 200–600 fs region a *silent state*. In the tumbling state, the intensity reduction of C=C stretching is not as drastic as those of the HOOP and in-plane bending, as the number of the C=C stretching modes is larger than that of the C–C–H configuration and the C=C stretching is less sensitive to the torsion angle than the HOOP and in-plane bending modes. These features can be explained by the torsion motion of the two moieties of the retinal Schiff base separated by the $C_{13}=C_{14}$ double bond just after excitation. The torsion motion and the broadening due to the inhomogeneous distribution of the torsion angles and the rapid change in frequencies induce the reduction of the intensities of the corresponding vibrational bands.

The final event occurs 700 fs later. At 700–900 fs, the intensities of the in-plane C=C–H bending and C–C stretching modes (1150–1250 cm^{-1}) are partially recovered due to the narrowing of the torsion angle distribution in the quasi-thermal state after relaxing to the bottom of the potential curve of the A_g-like excited state along the torsion angle coordinate. After this relaxation to the ground state, the

conformational change takes place again through LZ tunneling to the 13-*cis* conformation, *i.e.* K_{610}, with 55% efficiency (23). This verifies that the oscillating signal is not caused by the molecular vibration in the ground electronic state but by vibration in the excited states. The gate delay time of 700–800 fs corresponds to the period of the population thermalized before it is converted to K intermediate.

We further studied the modulation of C=C stretching frequency associated with the torsion mode. When we utilize the empirical equation representing the relation between the bond length and frequency (34), the change of frequency is estimated to be related to change in the bond length of $C_{13}=C_{14}$ double bond. The equation is

$$v = C_i\left[a(P + 1)(\chi/d)^{3/2} + b\right]^{1/2}$$

where $a = 1.67 \times 10^5$, $b = 0.30 \times 10^5$, $\chi = 2.5$, $C_i = 1.66$, $d = 1.489 - 0.151P$.

When the carbon bond is single bond or a double bond, P is 0 and 1, respectively. The observed change of frequency of the C=C stretching mode revealed that the bond length of $C_{13}=C_{14}$ double bond was changed about 10 mÅ.

From these data, the relations among the real-time torsion angle, bond order, and change in the equilibrium distance between two carbon atoms composing $C_{13}=C_{14}$ double bonds can be discussed as shown in Fig. 2. Thus we could determine the C=C bond length during the torsion motion around the double bond with 1-fs resolution with about 0.01 Å accuracy and precision.

CONCLUSION

By using sub-5-fs visible laser pulses, we have made the first observation of the vibrational spectra of the transition state during *trans-cis* isomerization in a retinal chromophore of bR. No instant isomerization of the retinal occurs in spite of electron promotion from the bonding π-orbital to the anti-bonding π^*-orbital. The difference between the in-plane and out-of-plane vibrational frequencies (about 1150–1250 and 900–1000 cm^{-1}, respectively) is reduced during the first period. The vibrational spectra after this period becomes very broad and weak, and this is ascribed to a "*silent state*." The silent state lasts for 700–900 fs and then the chromophore isomerizes to the *cis*-$C_{13}=C_{14}$ conformation.

The frequency of the C=C stretching mode was modulated by the torsion mode of the $C_{13}=C_{14}$ double bond with a period of 200 fs. The modulation was clearly observed for four to five periods. From the real-time data, we discussed the relations among the real-time torsion angle, bond order and the change in the equilibrium distance between two carbon atoms composing the $C_{13}=C_{14}$ double bond. Using the empirical equation of the relation between bond length and stretching frequency, we determined the C=C bond length with about 0.01 Å accuracy during the torsion motion around the double bond with 1-fs time resolution.

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