Analysis of the Excited-State Dynamics of 13-*trans-locked*-Bacteriorhodopsin

Ryo Akiyama,[†] Akira Yoshimori, and Toshiaki Kakitani*

Department of Physics, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

Yasushi Imamoto

Department of Earth and Space Science, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan

Yoshinori Shichida

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Yasuyo Hatano

School of Computer and Cognitive Science, Chukyo University, Toyota, Aichi 470-03, Japan

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Optical absorption spectra of 13-*trans-locked*-bacteriorhodopsin, which contains a chemically modified retinal chromophore inhibiting photoisomerization, were obtained at five temperatures. Analysis of the excited-state dynamics of the time-correlation function (tcf) of the modified wavepacket was made by the Fourier transform of the optical absorption spectra. Even though the photoisomerization of the chromophore was inhibited, the normalized tcf decayed rapidly until the level of about 10^{-6} at 200 fs almost independently of the temperature. The ratio of the tcf between 13-*trans-locked*-bacteriorhodopsin and native bacteriorhodopsin displayed some oscillations. Its mean value was close to 1 until about 100 fs, and it increased gradually up to the level of $10^{0.5}-10^{1}$ at about 200 fs. Namely, the excited-state dynamics of 13-*trans-locked*-bacteriorhodopsin appears globally quite similar to that of native bacteriorhodopsin up to about 100 fs, and the difference of them becomes slightly evident after 100 fs up to about 200 fs. Those data suggest that the excited-state dynamics of bacteriorhodopsin is not solely determined by the conformation change of the chromophore but also by another factor such as the movement of the microenvironment of the protein.

Introduction

Photosynthesis in *Halobacterium salinarium* is based on the photoisomerization of a chromophore in the retinal proteins.[1,2] There are two kinds of light-driven ion pumps for energy generation in the bacteria. One is bacteriorhodopsin, and another is halorhodopsin. Bacteriorhodopsin has the function of a proton pump from cytoplasmic to the extracellular side and creates an electrochemical proton gradient which is used for adenosine triphosphate production.

In bacteriorhodopsin, the photoisomerization, which takes place from the all-trans form to the 13-cis form of the chromophore, is very fast. Many kinds of spectroscopic studies have been done for this ultrafast phenomenon.[3−18] According to them, the excited-state dynamics ceases in about 200 fs and the primary intermediate of the ground state is produced within 500 fs after the excitation.[10−12]

In order to elucidate the mechanism of this ultrafast photoisomerization, two kinds of comparative studies were useful. One is the substitution or modification of the environment for the chromophore, and another is the modification of the chromophore itself. In the case of alternation of the environment, it was found that the photoisomerization of the chromophore in the protein environment of bacteriorhodopsin is faster than that of the all-trans protonated retinylidene Schiff base in methanol by the study of time-resolved fluorescence spectroscopy.[13] Replacement of functionally important charged and neutral amino acid residues by the site-directed mutagenesis caused a substantial decrease of the decay of the population in the excited state resulting from the formation of the J intermediate.[21]

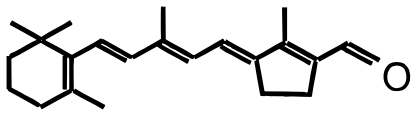

Figure 1. Molecular structure of 13-*trans-locked*-retinal.

On the other hand, some experiments have been carried out to examine the effect of the modification of the chromophore itself on the isomerization rate.[14−18] For example, *all-trans*-retinal is substituted by a six-membered ring spanning the C_9-C_{11} positions of the polyene chain (Ret6.9),[14] a six-membered ring spanning the $C_{11}-C_{13}$ positions of the polyene chain (Ret6.11),[15] or a five-membered ring spanning the $C_{12}-C_{14}$ positions of the polyene chain (Ret5.12, which we call 13-*trans-locked*-retinal in this paper, and its molecule structure is shown in Figure 1).[16] Artificial bacteriorhodopsins incorporating those chromophores were examined by time-resolved fluorescence spectroscopy and/or time-resolved absorption and resonance Raman spectroscopy. The artificial bacteriorhodopsins which contain Ret6.9 or Ret6.11 produced J- and K-like intermediates without much change in the kinetics.[14,15] The artificial bacteriorhodopsin which contains 13-*trans-locked*-retinal, which we call 13-*trans-locked*-bacteriorhodopsin hereafter, did not produce the K-like intermediate. Namely, the photoisomerization did not take place. However, the red-shifted intermediate called the T intermediate was transiently formed.[16]

[†] Present address: Institute for Molecular Science.

* All the correspondence should be addressed to Prof. T. Kakitani. Phone and Fax: +81-52-789-3528. E-mail: kakitani@allegro.phys.nagoya-u.ac.jp.

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Alternatively, excited-state dynamics in a molecular system has also been studied by an indirect method. It is a Fourier transform of the optical absorption spectrum (FTOA) method.[17,18,22−25] This method gives us a time-correlation function (tcf) of the vibrational wavepacket propagation modified by the transition dipole moment in the excited state. The method is suitable for the study of ultrafast femtosecond excited-state dynamics, because the accuracy of the tcf is much better at early times than at later times. This method has been applied to benzene solutions,[22,23] rhodopsin,[24,25] and bacteriorhodopsin.[17,18] Especially, the temperature dependence of the excited-state dynamics of bacteriorhodopsin has been extensively investigated.[17,18]

Recently, theoretical analysis of the temperature dependence of the tcf of bacteriorhodopsin was made by the spin-boson model,[18] in which motions of the environment of the chromophore are also assumed to be harmonic oscillators, and this model was useful for analyzing the mechanism of the electron-transfer reaction in solution and protein environment.[19,20] It was shown that the spin-boson model is valid to reproduce the remarkable temperature dependence of the very rapid decay of the tcf only in the short time region of less than about 30 fs and that the tcf in the time region larger than 30 fs is dominated by the nonharmonic slow vibrational motion, which is nearly temperature independent.[18] It was also suggested that the latter specific vibrational motions might reflect the ultrafast cis−trans isomerization of the chromophore as well as the conformation change of the protein environment, apparently free from the inhomogeneous broadening effect which, if exists, contributes to decay the tcf very rapidly at larger times.[17]

In this paper, we obtain the tcf of 13-*trans*-*locked*-bacteriorhodopsin by the Fourier transform of the optical absorption spectra at five temperatures and compare it with the tcf of native bacteriorhodopsin. The motive force of this study is to see how the excited-state dynamics is affected by locking the bond which makes photoisomerization. A simple expectation is that the excited-state dynamics will become much limited, as compared with the native case.

Material and Methods

1. Preparation of 13-*trans*-*locked*-Bacteriorhodopsin. The purple membrane was isolated from cultured cells of *Halobacterium salinarium* strain ET1001 according to the method previously reported,[28] and the 13-*trans*-*locked*-retinal was synthesized as reported previously.[29] For the preparation of bacterioopsin used for the reconstitution with 13-*trans*-*locked*-retinal, bacteriorhodopsin in the purple membrane was completely bleached by irradiation with >500-nm light for about 20 h at 293 K in the presence of 2 M hydroxylamine. The bleached membrane was washed 5 times with 2% BSA (bovine serum albumin) solution by centrifugation to remove hydroxylamine and retinaloxime. After removal of BSA by washing 5 times with the buffer, about 2-fold M excess 13-*trans*-*locked*-retinal dissolved in 10 μL of ethanol was added to the bleached membrane sample (15 mL), followed by incubation for 14 days at room temperature. The membrane was washed 3 times with 2% BSA solution to remove the excess 13-*trans*-*locked*-retinal and then washed 5 times with the buffer. The purple membrane containing 13-*trans*-*locked*-bacteriorhodopsin was suspended in the 66% glycerol buffer (5 mM Na, potassium phosphate, 100 mM NaCl, 66% glycerol, pH 6.5) and sonicated (1 min 3 times, HEAT SYSTEM-ULTRASONICS, W-220) to reduce the turbidity of the sample.

2. Spectroscopy. Absorption spectra were recorded with a Shimadzu MPS-2000 spectrophotometer equipped with an optical cryostat (Oxford, CF1204) connected with a temperature controller (Oxford, ITC4). The scanning speed and the spectral slit width of the probe beam were set at "slow" and 0.5 nm, respectively. At each temperature, 50 absorption spectra of the 13-*trans*-*locked*-bacteriorhodopsin sample or 66% glycerol buffer were recorded at 0.2-nm intervals and averaged, followed by calculation of their difference spectra to obtain the precise absorption spectrum of 13-*trans*-*locked*-bacteriorhodopsin. To record the absorption spectra at 273, 233, and 193 K, at which no cracks were formed on cooling, the sample or 66% glycerol buffer was put into a quartz cell (light path length = 2 mm) and cooled in the cryostat. For recording the absorption spectra at 133 and 78 K, the sample or 66% glycerol buffer was rapidly injected to the cell (light path length = 2 mm) precooled by liquid nitrogen using a syringe (rapid cooling technique[30]) to obtain the transparent sample without cracks.

3. Method. A Fourier transform of an optical absorption spectrum gives a tcf $\langle\phi(0)\cdot\phi(t)\rangle$ which is defined by

$$\langle\phi(0)\cdot\phi(t)\rangle = \int_{-\infty}^{\infty} \frac{I(\omega)}{\omega} \exp(i\omega t)\, \mathrm{d}\omega \tag{1}$$

where the function $I(\omega)$ is the optical absorption spectrum and ω is the angular frequency.[23,26,27] The modified wavepacket, $\phi(t)$, is defined as

$$\phi(0) = \hat{\mu}_{if}\chi_i \tag{2}$$

$$\phi(t) = \exp\left(\frac{i\hat{H}t}{\hbar}\right)\phi(0)\exp\left(\frac{-i\hat{H}t}{\hbar}\right) \tag{3}$$

where χ_i is the vibrational wave function in the ground state, $\hat{\mu}_{if}$ the transition dipole moment, and $\hat{H}$ the vibrational Hamiltonian in the excited state. In eq 1, the function $\phi(0)$ means the modified wavepacket at the Franck−Condon state and the function $\phi(t)$ means the propagating modified wavepacket at time t. Here, we define the normalized tcf $C(t)$ as follows:

$$C(t) = \frac{\langle\phi(0)\cdot\phi(t)\rangle}{\langle\phi(0)^2\rangle} \tag{4}$$

The normalized tcf $\langle\mathbf{u}(0)\cdot\mathbf{u}(t)\rangle$ defined previously[17,24] corresponds to this $C(t)$. The phase $\Theta(t)$ is defined by

$$\tan\Theta(t) = \frac{\mathrm{Im}\ C(t)}{\mathrm{Re}\ C(t)} \tag{5}$$

where Im and Re indicate the imaginary and real parts, respectively. From these definitions, the absolute value of $C(t)$ can be used as a measure to see how much conformation change occurs after excitation as a function of time.[23] The phase $\Theta(t)$ picks up only the coherent part of the conformation change and reflects the potential energy height which the wavepacket sees at each time.[23] We calculate $C(t)$ from the experimentally obtained absorption spectrum by the same method as before.[17,24,25]

Results

1. Optical Absorption Spectra of 13-*trans*-*locked*-Bacteriorhodopsin. In Figure 2a, we show the optical absorption spectrum of 13-*trans*-*locked*-bacteriorhodopsin observed at five temperatures: 78, 133, 193, 233, and 273 K. At 78 K, the wavelength of the maximum absorption, λ_{max}, of the α band is 587 nm, and its value changes little at higher temperatures. The absorption spectra at 78, 133, and 193 K have remarkable humps

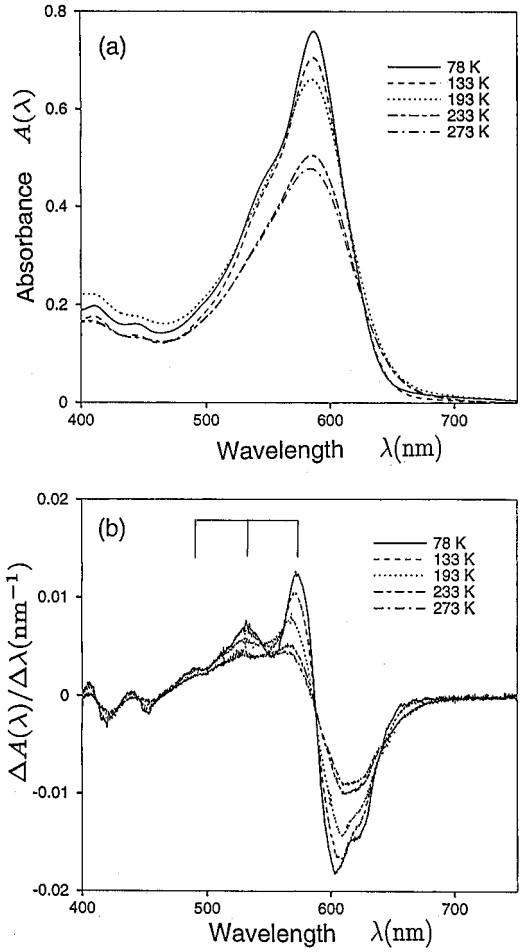

Figure 2. Experimental data of (a) the optical absorption spectra (50 times accumulated) and (b) those derivative functions ($\Delta\lambda = 0.2$ nm) at 78, 133, 193, 233, and 273 K. Corrections of the volume-contraction effect at low temperatures are not made.

at the shorter wavelength side of λ_{max}. Figure 2b shows those derivatives of the spectra.

Remarkable structures are seen at the shorter wavelength side from λ_{max} as well as two negative peaks at the longer wavelength side. The peak separations in the region from 450 to 600 nm are 42 and 40 nm. These separations correspond to the vibration of about 1300 cm^{-1}. These peak separations remain nearly the same for all the temperatures, although the peak height becomes much more decreased as the temperature increases.

In the native bacteriorhodopsin, the peak separation in this wavelength region was about 1400 cm^{-1} for 78 K and 690 cm^{-1} for 273 K.[17] Therefore, the presently obtained peaks for 13-*trans-locked*-bacteriorhodopsin roughly correspond to those of native bacteriorhodopsin at 78 K.

2. Time Correlation Functions. In Figure 3, we show the absolute value of the normalized tcf $|C(t)|$ for 13-*trans-locked*-bacteriorhodopsin. Unexpectedly, those curves decayed very rapidly with time. A general feature of $|C(t)|$ is summarized as follows. The curve is classified into three parts. In the time region up to 20−40 fs, the curves decrease very rapidly from 1 to 10^{-2}−10^{-3}. In the region from 20−40 fs up to 200−230 fs, the tcf decreases more slowly with evident structures to 10^{-6}−10^{-7}. After this time, those curves decrease more slowly and monotonously. The temperature dependence of $|C(t)|$ is summarized as follows. First, the initial decrease (≤40 fs) is more rapid at higher temperatures. And in the time region from 20−40 fs to 200−230 fs, those decreases have no evident temperature dependence. All these global features of $|C(t)|$ are similar to those of native bacteriorhodopsin.[17]

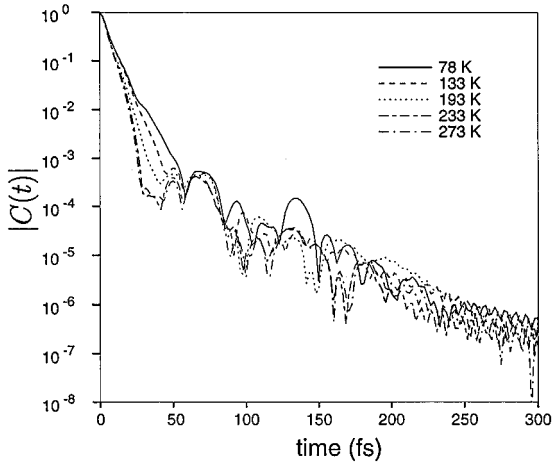

Figure 3. Calculated values of $|C(t)|$ of 13-*trans-locked*-bacteriorhodopsin in the time range up to 300 fs superimposed for 78, 133, 193, 233, and 273 K.

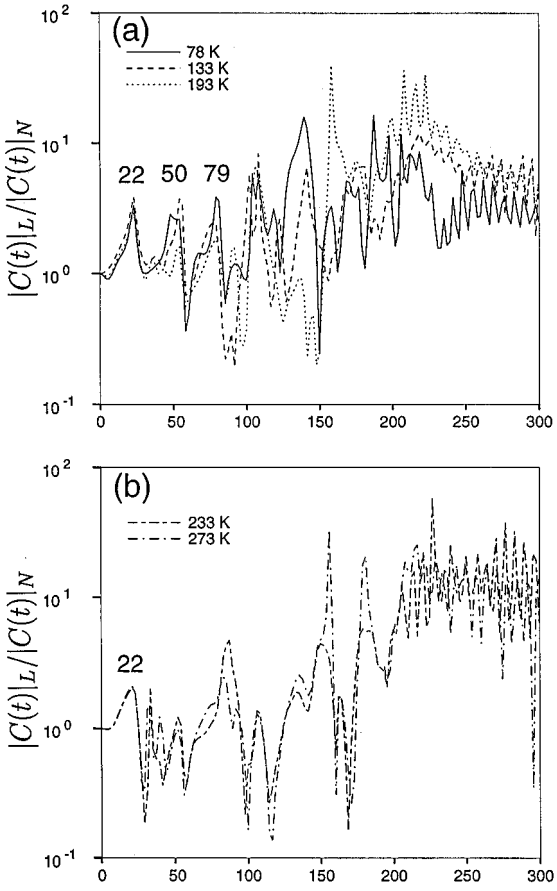

Figure 4. Ratio of $|C(t)|_L/|C(t)|_N$ in the time range up to 300 fs superimposed for (a) 78, 133, and 193 K and (b) 233 and 273 K.

However, if we compare them in detail, we see some differences. We calculated the ratio of $|C(t)|$ between 13-*trans-locked*-bacteriorhodopsin and native bacteriorhodopsin at the same temperature ($|C(t)|_L/|C(t)|_N$). Figure 4a shows the ratio at three low temperatures (78, 133, and 193 K), and Figure 4b shows the ratio at two high temperatures (233 and 273 K). Although these curves fluctuate very much, we can derive some properties as follows. In the early time region up to about 100 fs, systematic peaks appear at 22, 50, and 79 fs for lower temperatures (Figure 4a) and the corresponding peak at 22 fs for higher temperatures (Figure 4b). After 100 fs, the mean value of the ratio appears to increase gradually until about 200 fs by a factor of about $10^{0.5}$ at 78, 133, and 193 K and by a

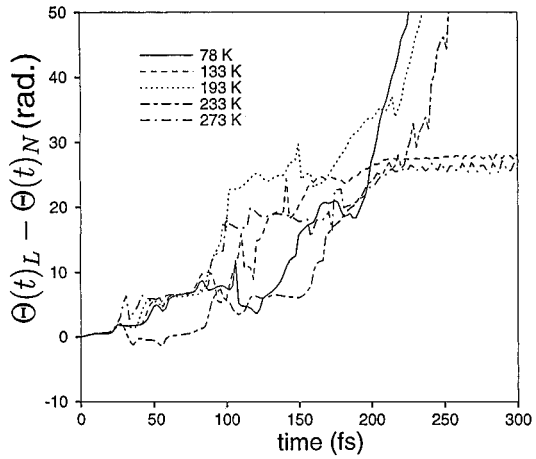

Figure 5. Difference of the phase $\Theta(t)_L - \Theta(t)_N$ in the time range up to 300 fs superimposed for 78, 133, 193, 233, and 273 K.

factor of about 10^1 at 233 and 273 K. It is important to note that this global change in the time region from 100 to 200 fs is brought about by inhibiting the isomerization of the chromophore.

The difference of the phase of $C(t)$ between 13-*trans-locked*-bacteriorhodopsin and native bacteriorhodopsin ($\Theta(t)_L - \Theta(t)_N$) is plotted in Figure 5. Although its curve differs considerably among five temperatures, it might be due to noise (since the coherent part of conformation changes is immersed in incoherent ones, the accuracy of the phase is low). If we take those averages, we can say that the phase difference globally increases slowly until 100 fs and then increases more rapidly until 200 fs. After 200 fs, the curves diverge too much to take an average.

The derivative $d\Theta/dt$ roughly corresponds to the difference between the energy level of the wavepacket in the excited-state potential surface and the zero vibrational energy level in the ground state.[23] Therefore, the positive slope of $\Theta(t)_L - \Theta(t)_N$ indicates that the potential energy curve of the excited state of 13-*trans-locked*-bacteriorhodopsin is higher than that of native bacteriorhodopsin. So the data in Figure 5 indicate that the excited-state dynamics represented by the motion of the wavepacket in 13-*trans*-bacteriorhodopsin is restricted step by step, compared with that of native bacteriorhodopsin.

Discussion

In our previous paper,[17] we calculated the function $|C(t)|$ by using experimental data of vibrational wavenumbers and displacements of normal coordinates for 29 harmonic modes and using the theory of the simple harmonic oscillator model. The experimental data were those obtained by the analysis of the resonance Raman line for bacteriorhodopsin at room temperature.[4] The calculated value of $|C(t)|$ oscillated with a period of about 23 fs up to about 100 fs and approached a constant value of 0.53 (about $10^{-0.5}$) at the later time.[17] The experimental data of $|C(t)|$ decreased rapidly down to the level of $10^{-3}-10^{-4}$ until about 40 fs and then decreased with slower speed down to the level of about $10^{-6}-10^{-7}$ until about 200 fs. This great difference between the theoretically calculated curve of $|C(t)|$ and the experimental data was thought to be due to the inhomogeneous broadening effect and/or due to the unknown vibrational mode. When we incorporated a considerable amount of the inhomogeneous effect by the Gaussian or Lorentzian form, the corrected tcf decreased rapidly down to $10^{-3}-10^{-4}$ until about 40 fs, in agreement with the experimental data, but it continued to decrease with the same or rapider speed after 40 fs, in disagreement with the experimental data. Therefore, we anticipated that the inhomogeneous broadening effect might not play a predominant role in $|C(t)|$ at least in the time region larger than 40 fs and that some motions might exist which have very large displacements of potential minima between the ground and excited state corresponding to the isomerization of the chromophore, which were not observed by the resonance Raman experiment.[17,25]

If this anticipation is right, we can expect that the decay of the tcf is much decreased when the photoisomerization was inhibited. However, in the present study, we showed that the decay of $|C(t)|$ resembles that of native bacteriorhodopsin as a whole. Namely, even though the isomerization of the chromophore is inhibited, the value of $|C(t)|$ approached about $10^{-3.5}$ at 40 fs and 10^{-5} at 150 fs (see Figure 3). Therefore, a large decrease of tcf is not dominantly caused by the isomerization coordinate. Of course, there is a possibility that bonds other than C_{13}═C_{14} bond are twisted to some extent by the photoexcitation in 13-*trans-locked* bacteriorhodopsin. However, it is not expected that the effect of such conformation change upon the tcf is very similar to the case of native bacteriorhodopsin at least up to 100 fs, as seen in Figure 4.

In Figure 4, we found systematic peaks at 22, 50, and 79 fs in the ratio of tcf. The peak is always larger than one. This indicates that either some mode with a period of 22−29 fs disappeared by a lock of the C_{13}═C_{14} bond twisting of the chromophore or the oscillating amplitude of the mode with a period of 22−29 fs is decreased by a lock of the C_{13}═C_{14} bond twisting. We can resolve this problem by using Figure 2. We saw the progression of peaks with interval of about 1300 cm^{-1} in Figure 2b. The period of this oscillation is about 23 fs, which might correspond to the above separation of peaks in the ratio. If we see the relative amplitude of the progression in Figure 2b, it monotonically decreases as it goes to the shorter wavelength side. In contrast to this, in the native bacteriorhodopsin (Figure 2 of ref 17), the relative amplitude increases once and then decreases as it goes to the shorter wavelength side. This fact indicates that the 22−29 fs period mode exists in both 13-*trans-locked*-bacteriorhodopsin and native bacteriorhodopsin, and the magnitude of the displacement of the equilibrium point after excitation is smaller in 13-*trans-locked*-bacteriorhodopsin than native bacteriorhodopsin (namely, the Franck−Condon facter of the 22−29 fs period mode is smaller in the former), supporting the idea that the oscillating amplitude of this mode in the tcf is decreased by a lock of the C_{13}═C_{14} bond twisting.

We have seen in Figure 4 that the mean value of the ratio of tcf remains the same until 100 fs and increases by 1 order in the time range 100−200 fs at higher temperatures. This fact would indicate that after about 100 fs, some kind of motion of bacteriorhodopsin was reduced by the lock of the C_{13}═C_{14} bond twisting.

In Figure 5, we saw that the difference of the phase increases slowly up to 100 fs and then it increases more rapidly. Since the change of the phase reflects a change of coherent motion of vibrations, we can expect that there is some mode which moves slowly until 100 fs and moves actively after 100 fs until 200 fs.

On the basis of the above comparative study between 13-*trans-locked*-bacteriorhodopsin and native bacteriorhodopsin, we are led to a working hypothesis or model that a considerable part of the conformation change of bacteriorhodopsin after the photoexcitation which contributes to the tcf comes from the motion other than the chromophore, namely, movement of residues such as charged or polar groups around the chromophore, which may be called microenvironmental motions of protein. It is expected that this microenvironmental movement

of protein will take place cooperatively with the conformation change of the chromophore due to photoexcitation. By a suitable design of the microenvironment, it may become possible that the photoisomerization of the chromophore in bacteriorhodopsin is faster than that is solution, as experimentally observed.[13−15]

Let us ask what is the mechanism by which these protein modes couple to the electronic transition. According to the quantum chemical calculations for the protonated Schiff base of 11-*cis*-retinal and *all*-*trans*-retinal, the charge distribution is shifted largely from the Schiff base side to the ionone ring side by the photoexcitation.[31,32] Therefore, it will be possible that some charged or polar groups of protein environment near the Schiff base or the ionone ring begin to move, following a sudden charge redistribution of the chromophore due to photoexcitation. If one of those charged amino acid residues is substituted by a neutral amino acid residue, the photoisomerization rate can be affected. Indeed, Song *et al*. reported that decay rates of the excited state of bacteriorhodopsin were largely decreased by the point mutation of D85N, R82Q, or D212N.[21] One of possible explanations of this result might be that the amount of movement of the 85th, 82th, or 212th charged amino acid residue in the excited-state dynamics of bacteriorhodopsin is reduced by the mutation due to a weakened electrostatic coupling between the chromophore and the substituted neutral amino acid residue.

Apparently, it is strange that $|C(t)|$ of the 13-*trans*-*locked*-bacteriorhodopsin increased by only 1 order of magnitude at most in 200 fs, compared with that of native bacteriorhodopsin. If the C_{13}═C_{14} bond of the chromophore in native bacteriorhodopsin is twisted by about 90° at 200 fs, in which the transition from the excited state to the ground state might start to take place, a bigger difference of $|C(t)|$ can be expected between the modified and native bacteriorhodopsins at 200 fs. To resolve this mystery, we have the following notion. In the course of photoisomerization of the chromophore of native bacteriorhodopsin, not only the C_{13}═C_{14} bond but also the other bonds of the chromophore will be twisted simultaneously by keeping the global form similar to that of the Franck−Condon state in the binding pocket, as suggested by Warshel.[33] Since the C_{13}═C_{14} bond is not allowed to twist in 13-*trans*-*locked*-bacteriorhodopsin, the twistings of the other bonds of the chromophore must be adjusted in the excited-state dynamics in order to keep the global form of the chromophore similar to that of the Franck−Condon state as much as possible. In such a way, the global conformation of the chromophore would not differ so much even at 200 fs between the modified and native bacteriorhodopsins. This might be the reason why $|C(t)|$ does not differ so much. Since the C_{13}═C_{14} bond is not twisted and the twistings of the other bonds might be enhanced in 200 fs in the modified bacteriorhodopsin, the conformation of the protein environment might also be altered to some extent. Under such situation, it is reasonable to consider that the red-shifted intermediate named the T intermediate[16] is produced in the cource of the relaxation on the potential hill after the nonradiative transition to the ground state. Since 13-*trans*-*locked*-bacteriorhodopsin is recovered in 17 ps after photoexcitation,[16] the possibility of isomerization of bonds other than the C_{13}═C_{14} bond will be neglected.

Finally, we mention the inhomogeneous broadening which might appear when the protein conformation is largely distributed due to the trap at different local minima in the potential energy surface. As we stated in the Introduction, the systematic, remarkable temperature dependences of $|C(t)|$ of bacteriorhodopsin up to about 30 fs were successfully reproduced using the weighted density of state obtained with aid of the spin-boson model.[18] The similar analysis was made for the temperature dependence of $|C(t)|$ of 13-*trans*-*locked*-bacteriorhodopsin.[34] Then, we found that the spin-boson model is useful for reproducing this temperature dependence of $|C(t)|$ also up to about 30 fs.[34] We also found that the vibrational modes with wavenumbers larger than about 160 cm^{-1} in the weighted density of states essentially determined the rapid decay of $|C(t)|$ until about 30 fs and the nonharmonic slow vibrational motions dominate the slow decay of $|C(t)|$ after 30 fs.[18,34] On the basis of these results, we may say that even if the inhomogeneous broadening effect exists in the optical absorption spectrum, its effect either is small or is a much restricted one which has the same temperature dependence of $|C(t)|$ as the harmonic oscillators until about 30 fs and the nonharmonic character after 30 fs.

Conclusion

As the conclusion of this paper, by means of the FTOA analysis, we found that the tcf of the wavepacket propagation in the excited state of 13-*trans*-*locked*-bacteriorhodopsin is globally similar to that of native bacteriorhodopsin, including those temperature dependences. This result indicates that the excited-state dynamics of bacteriorhodopsin is not solely determined by the conformation change of the chromophore but also by another factor such as the movement of the microenvironment of protein. When we take the ratio of the absolute value of tcf and the difference of the phase between the above two kind of bacteriorhodopsins, specific differences of the wavepacket motions between them are obtained. Namely, we found that in the shorter time region, the motion of some high-frequency mode ($\sim$ 1300 cm^{-1}) is restricted and at times later than 100 fs, some kinds of slow motions of chromophore or microenvironment of protein are restricted as an effect of forbidding the C_{13}═C_{14} bond twisting.

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