A Spectrally Silent Transformation in the Photolysis of Octopus Rhodopsin: A Protein Conformational Change Without Any Accompanying Change of the Chromophore’s Absorption

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ABSTRACT A spectrally silent transformation in the photolysis of octopus rhodopsin was detected by the time-resolved transient grating method. Our results showed that at least two photointermediates, which share the same chromophore absorption spectrum, exist after the final absorption changes. Previously, mesorhodopsin was thought to decay to the final photoproduct, acid metarhodopsin with a lifetime of 38 μs at 15°C, but the present results show that there is at least one intermediate species (called transient acid metarhodopsin) with a lifetime of 180 μs at 15°C, before forming acid metarhodopsin. This indicates that the parts of the protein distant from the chromophore are still changing even after the changes in microenvironment around the chromophore are over. From the signal intensity detected by the transient grating method, the volume change of the spectrally silent transformation was found to be ΔV = 13 ml/mol. The activation energy of the spectrally silent transformation is much lower than those of other transformations of octopus rhodopsin. Since stable acid metarhodopsin has not been shown to activate the G protein, this transient acid metarhodopsin may be responsible for G protein activation.

INTRODUCTION

Rhodopsins consist of 11-cis retinal chromophore covalently attached to the opsin via a protonated Schiff base linkage. Light isomerizes the 11-cis retinal to its all-trans form which initiates a series of protein conformational changes, called the photointermediates of bleaching, each usually characterized by their different absorption spectra. One of the photointermediates interacts with the G protein, resulting in the electrical excitation of a photoreceptor cell (Stryer, 1986; Tsuda, 1987). Although the photoreaction processes of both vertebrate and invertebrate rhodopsins resemble each other for several μs after photoexcitation, eventually they begin to differ. After metarhodopsin II, which activates the G protein, the vertebrate rhodopsins are finally hydrolyzed to free all-trans retinal and opsin. On the other hand, photolysis of the invertebrate pigments leads to a stable species containing all-trans retinal. In octopus rhodopsin, the stable acid metarhodopsin can be converted back to the original rhodopsin upon orange-light illumination (Tsuda, 1979, 1987). In a previous paper, Nakagawa et al. (1998) showed that stable final photoproduct could not activate the G protein, but a novel intermediate that lies between mesorhodopsin and stable acid metarhodopsin is a candidate for the species that can activate the G protein. Spectroscopic methods have been extensively applied to the study of the reactions following light excitation (Tsuda, 1979; Tsuda et al., 1980; Pande et al., 1987; Ohtani et al., 1988; Bagley et al., 1989; Deng et al., 1991; Taiji et al., 1992; Masuda et al., 1993; Nakagawa et al., 1997; Nishimura et al., 1997). Transient absorption spectroscopy showed that transformation of mesorhodopsin to acid metarhodopsin (with a lifetime of 31 μs at 15°C) is the final spectral transformation in the photolysis of octopus rhodopsin. Previously, Nakagawa et al. (1998) studied the photointermediate of octopus rhodopsin responsible for G protein activation by a GTPγS binding assay and concluded that a novel intermediate between mesorhodopsin and stable acid metarhodopsin, but not stable acid metarhodopsin, is the most likely candidate for the activating species. The presence of a precursor to stable acid metarhodopsin was suggested by transient absorption changes at alkaline conditions (Tsuda 1979), Fourier-transform infrared spectroscopy (FTIR) (Masuda et al., 1993) and light scattering measurements (Nakagawa et al., 1998). However, this postulated intermediate has not been yet identified kinetically. To study kinetics of a process which is not accessible by any optical absorption changes, the transient grating technique (Richard et al., 1992; Hara et al., 1996; Takeshita et al., 2000) or the photoacoustic technique (van Brederode et al., 1995) could be powerful methods; they monitor the reaction volume change. We examined the kinetics of the activation of octopus rhodopsin by using the time-resolved transient grating technique and found a spectrally silent transition accompanying the protein structure changes after the absorption changes are complete. Based on these investigations, formation of the acid metarhodopsin, which has previously been assigned by absorp-
tion spectroscopy, is shown in this paper to be the formation of transient acid metarhodopsin.

EXPERIMENTAL

Octopus rhodopsin was prepared as described previously (Tsuda et al., 1986). Briefly, microvillar membranes from the octopus retina (Octopus dofleini) were isolated by sucrose floatation and then washed with buffer A [400 mM KCl, 10 mM MgCl2, 10 mM 2-(N-morpholine)ethanesulfonic acid (pH 6.5), 1 mM dithiothreitol, 1 mM benzamidine-HCl, 20 μM 4-(amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF)] and then with buffer B [10 mM 4-morpholinepropanesulfonic acid (pH 6.5), 1 mM dithiothreitol, 1 mM benzamidine-HCl, 20 μM APMSF]. After the microvillar membranes were solubilized in 1% (w/v) sucrose monolaurate (SM 1200), 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM dithiothreitol, 1 mM benzamidine-HCl, and 20 μM APMSF, octopus rhodopsin was affinity purified by concanavalin-A (Con-A) sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). Finally, the sample (~80 μM rhodopsin) for the transient grating and transient absorption measurements was prepared in 10 mM 3-(N-morpholinopropanesulfonic acid (pH 7.4) and 1% SM 1200.

The transient grating setup was similar to that previously described (Terazima and Hirota, 1993; Hara et al., 1996). Briefly, the beam of a XeCl eximer laser-pumped dye laser (Lambda Physik Compex 102xc, Lumomics Hyper Dye 300; λ, 465 nm, Göttingen, Germany) was divided by a beam splitter and the beams crossed inside a quartz sample cell (optical path-length, 2 mm). The laser power of the excitation was <8 μJ/pulse. The interference pattern (transient grating) created in the sample was probed by a He-Ne laser (633 nm) or a diode laser (840 nm) as a Bragg diffracted signal (transient grating signal). The transient grating signal was detected by a photo-multiplier (Hamamatsu R928, Iwata, Japan) and averaged by a digital oscilloscope (Tektronix 2430A, Toyko, Japan). A transient absorption signal was also detected by a photo-multiplier (Hamamatsu R928, Iwata, Japan) and averaged by a digital oscilloscope using a nanosecond laser pulse, one of the dominant contributions is the temperature change of the medium induced by the energy from the radiationless decay of excited states and by the enthalpy change of the reaction (thermal grating δn(th)(t)). Another important contribution originates from the change in the absorbance of the solution induced by light. The newly formed species and the depletion of the original species contribute to the gratings through the absorption changes. We have called this component the population grating (δn(pop)(t)). The partial molecular volume (V) difference between the reactant and products (ΔV) induces a change of the average density of the medium, and this change causes the refractive index change δn.vol (volume grating). In this work, the term “species grating” is used to describe both of these contributions (δn(spec) = δn(pop) + δn.vol). If the contribution of absorbance is negligible and the diffraction efficiency of the probe light is small, the transient signal intensity is proportional to the square of the peak-null difference of the refractive index created by the interference pattern of the excitation light intensity (Eichler et al., 1986; Miller, 1989; Terazima and Hirota, 1993; Hara et al., 1996).

\[ I_{TG}^{1/2}(t) = A[δn_{th}(t) + δn_{pop}(t) + δn_{vol}(t)] \] (1)

where A is a constant. The refractive index change of the volume grating component is given by

\[ δn_{vol} = V \frac{dn}{dV} ΔV ΔN \] (2)

where Vdn/dV is the refractive index change by the molecular volume change, and ΔN is the number of the reacting molecules in a unit volume. If the protein structure changes, the dynamics should be apparent in the transient grating signal through this component (even if the optical absorption spectrum does not change). By a quantitative measurement of δn.vol and the known solvent properties (Vdn/dV), we can determine the absolute value of ΔV.

The temporal profile of the refractive index grating due to the thermal grating is given by

\[ δn_{th}(t) = (dn/dt)ρC_p[Q(t)dt*exp(−D_θq^2t)] \] (3)

where * is the convolution integral, Q(t) is the thermal energy coming out from the sample, dn/dT is the temperature dependence of the refractive index, ρ is the density, C_p is the heat capacity at a constant pressure, and D_θ is the thermal diffusivity. The time dependence of the species grating is determined by the kinetics of the reaction and the molecular diffu-
sion process. If we can neglect the reaction kinetics in the molecular diffusion time-region, the time dependence is given by (Terazima and Hirota, 1993; Hara et al., 1996)

$$\delta n_{\text{spe}}(t) = \delta n_r \exp(-D_r q^2 t) + \delta n_p \exp(-D_p q^2 t)$$  \hspace{1cm} (4)

where $q$ is a grating wave number, and $\delta n_r$ and $\delta n_p$ represent refractive index changes by the reactant and product, respectively. $D_r$ and $D_p$ are the mass diffusion coefficients of the reactant and product, respectively. Because the magnitude of $D_{th}$ is ~2–3 orders of magnitude larger than $D_r$ and $D_p$, we can easily separate the thermal and species grating contributions (Hara et al., 1996; Takeshita et al., 2000). Furthermore, the reaction kinetics can be separated from the diffusion process by measuring the transient grating dynamics at different $q^2$, because the diffusion process depends on $q^2$, while the reaction kinetics should not.

**RESULTS AND DISCUSSION**

Fig. 1 shows the time course of the transient absorption changes at 543.5 nm after photolysis at 15°C. The kinetics can be described with two lifetimes, 520 ns and 38 μs, at this probe wavelength. These processes have been attributed to the lumirhodopsin $\rightarrow$ mesorhodopsin $\rightarrow$ acid metarhodopsin transformations. The absorption changes were essentially complete within 100 μs. These observations are consistent with previous findings (Nakagawa et al., 1997). The species formed with 38 μs (or 31 μs as reported by Nakagawa et al., 1997) has previously been considered to be the final photoproduct and has been called acid metarhodopsin (Nakagawa et al., 1997). However, hereafter in this paper, we call it the transient acid metarhodopsin because we discovered new dynamics for the creation and decay of this species.

Fig. 2 shows the early time profile of the transient grating signal of the photolyzed octopus rhodopsin probed at 840 nm at 15°C. We found that the signal over this fast time scale ($t < 100 \mu$s) can be fitted by a sum of four exponential functions with lifetimes of 130 ns, 520 ns, 3.4 μs, and 38 μs at $q^2 = 2.0 \times 10^{12} \text{m}^{-2}$. One of the lifetimes, 3.4 μs, depends on $q^2$, but the others do not. Considering the reaction scheme (Scheme 1) and the lifetimes, we can identify the $q^2$-independent lifetimes to the bathorhodopsin $\rightarrow$ lumirhodopsin, lumirhodopsin $\rightarrow$ mesorhodopsin, and mesorhodopsin $\rightarrow$ transient acid metarhodopsin processes, respectively. These lifetimes agree well with those observed from the transient absorption measurements discussed just above, and support the reaction scheme (Nakagawa et al., 1997).

Besides these $q^2$-independent lifetimes, a $q^2$-dependent lifetime is observed. The $q^2$-dependent dynamic implies that this dynamic is governed by a diffusion process. It is easily assigned to a thermal diffusion process (eq. (3)), because the decay-rate constant agrees with $D_{th} q^2$ calculated from the reported $D_{th}$. Therefore, this signal is the thermal grating signal. It is created by the thermal energy from a nonradiative transition or an enthalpy change ($\Delta H$) accompanying the reaction. [This signal is not apparent under weak excitation conditions, but its intensity increases with increasing laser power (data not shown). The relative enhancement of the thermal grating intensity can be explained in terms of the multiphoton excitation of either rhodopsin or the intermediates within the excitation pulse width.] From the thermal grating signal intensity we can determine the enthalpies of these intermediates, and these results will be published elsewhere. Interestingly, even after the absorption changes are over, there are several components in the transient grating signal (Fig. 3). The longer time-temporal profile can be reproduced by three exponential functions, one with a lifetime of 180 μs and two with lifetimes that depend on $q^2$ (4.5 ms and 16 ms at $q^2 = 2.0 \times 10^{12} \text{m}^{-2}$). The slower $q^2$-dependent kinetics were assigned to the diffusion processes of rhodopsin and the final species in this time range (Eq. 4). The most interesting and important component is the one with the 180-μs lifetime. Because this rate does not depend on $q^2$, this
dynamic is not due to a molecular diffusion process. We will examine this component further in detail.

First, because the final photoproduct of octopus rhodopsin can be converted back to rhodopsin upon light irradiation, the excitation laser pulse may excite this photoproduct, which was created by the previous pulses and initiate the back reaction. In such a case, kinetics of intermediates in the back reaction could contribute to the transient grating signal. This possibility was excluded by transient grating measurements with different excitation repetition rates. We confirmed that the transient grating signal becomes weak, and almost disappears, by successive excitation with a faster repetition rate and without stirring the solution. Therefore, the contribution of the back reaction from the final product in the signal can almost be neglected. Secondly, with increasing laser power, the thermal grating signal intensity increases, whereas the species grating signal intensity is saturated. This implies that all of the species in the transient grating signal come from the photoexcitation of rhodopsin, and none come from the sequential multiphoton excitation of the rhodopsin. Thirdly, the origin of the signal was examined by changing the probe wavelength. The refractive index change due to the absorption spectrum change (population grating) depends on the probe wavelength as predicted by the Kramers-Kronig relationship (Longhurst, 1967), whereas the thermal grating and the volume grating intensities are less sensitive to the probe wavelength. If the refractive index change, which gives rise to the population grating signal, comes from an absorption change of the chromophore, the population grating signal intensity is expected to increase at shorter wavelengths (closer to the absorption bands). It was found that the relative intensities of the population grating signals, except for the 180-μs component, were indeed enhanced at 633 nm compared with those at 840 nm. Since the 180 μs component was less sensitive to the probe wavelength, the origin of this signal is not due to an absorption change of the chromophore, but due to a volume change. Since this component is silent for absorption spectroscopy, the component that induces the volume change originates from protein conformational changes without any accompanying absorption changes of the chromophore. From quantitative measurements of the signal intensity, we determined the volume change to be 13...
even after the changes in microenvironment around the protein distant from the chromophore are still changing. Fluorescence quenching experiments show that parts of the protein by a possible intermediate species.

A long-lived intermediate was found to exist before stable acid metarhodopsin by time-resolved Fourier-transform infrared spectroscopy (Masuda et al., 1993). During illumination of microvillar membranes by blue light, bands in the 1800 to 1700 cm\(^{-1}\) region changed, which could be associated with the change in the state of carboxylic group of Asp-81. A preliminary observation of a light-induced fluorescence quenching (Nakagawa and Tsuda, to be published) showed that the lifetime of the movement of the fluorescence probe attached at Cys345 in the C terminus of octopus rhodopsin was \(-1\) ms at 15°C.

The results of the present transient grating, FTIR and the fluorescence quenching experiments show that parts of the protein distant from the chromophore are still changing even after the changes in microenvironment around the chromophore are over. The observed kinetics (180 \(\mu\)s at 15°C) in the transient grating signal could represent the transformation from the transient acid metarhodopsin to the final product (acid metarhodopsin), or to another intermediate species, which should eventually transform to the acid metarhodopsin with a slower rate. We could examine whether there are other transient intermediate species with much longer lifetimes, or not, using the transient grating signal method on much longer time scale. However this longer time scale measurement is very difficult because the dynamics, which represent the molecular diffusion process, becomes dominant on such a later time scale. Thus, the species created with 180 \(\mu\)s is tentatively called acid metarhodopsin.

To further understand this process, we measured the transient grating signal at various temperatures from 0 to 35°C. Fig. 4 shows the kinetics of the transient grating signals probed at 840 nm for 0, 10.2, 24.8, and 33.9°C. From this temperature dependence of the rate (\(k\)), the enthalpy of activation (\(\Delta H^0\)) and the entropy of activation (\(\Delta S^0\)) were obtained from activated complex theory,

\[
\ln(kh/k_B T) = \Delta S^0/R - \Delta H^0/RT,
\]

where \(h\) is the Planck constant, \(k_B\) is the Boltzmann constant and \(R\) is the gas constant. Fig. 5 shows the Arrhenius plots of the formation rate of transient acid metarhodopsin together with the rates of the lumirhodopsin \(\rightarrow\) mesorhodopsin, and the mesorhodopsin \(\rightarrow\) transient acid metarhodopsin processes. Each of these kinetics can be described by the relation with a single \(\Delta H^0\). The \(\Delta H^0\) and \(\Delta S^0\) that we determined are listed in Table 1. It is interesting that, although \(\Delta H^0\) and \(\Delta S^0\) for the lumirhodopsin \(\rightarrow\) mesorhodopsin and mesorhodopsin \(\rightarrow\) transient acid metarhodopsin processes are similar, the values for the creation of acid metarhodopsin are much lower. The different values of \(\Delta H^0\) and \(\Delta S^0\) imply that the activation processes for lumirhodopsin \(\rightarrow\) mesorhodopsin and mesorhodopsin \(\rightarrow\) transient acid metarhodopsin are very different from those for the transient acid metarhodopsin \(\rightarrow\) acid metarhodopsin process. The kinetics of the lumirhodopsin to transient acid metarhodopsin form should reflect conformational changes of the protein moiety around the chromophore. Therefore, this different activation process is consistent with our conclusion that the structure change to acid metarhodopsin is not the dynamics around the chromophore.

In bovine rhodopsin, the formation of metarhodopsin II induces helix changes in the pigment so that this species can activate the G protein (Cohen et al., 1993). The structural change of octopus rhodopsin observed in this study may correspond to a similar change on this pigment. The low activation energy of the new dynamics probably reflects that this large amplitude motion is less hindered. The molecular volume expansion of 13 ml/mol may result from the deactivation process of the transient acid metarhodopsin to form acid metarhodopsin, or initially contacted area of the helix is open for the water, which may be responsible for the activation of the G protein by a possible intermediate species.

### Table 1

<table>
<thead>
<tr>
<th>Process</th>
<th>(E_m/\text{kJ mol}^{-1})</th>
<th>(\ln A)</th>
<th>(\Delta H^0/\text{kJ mol}^{-1})</th>
<th>(\Delta S^0/\text{J mol}^{-1} \text{K}^{-1})</th>
<th>(\Delta G^0/\text{kJ mol}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumi → meso</td>
<td>59.8</td>
<td>39.2</td>
<td>57.3</td>
<td>72.9</td>
<td>35.6</td>
</tr>
<tr>
<td>Meso → transient acid meta</td>
<td>79.5</td>
<td>43.3</td>
<td>77.1</td>
<td>107.3</td>
<td>45.1</td>
</tr>
<tr>
<td>Transient acid meta → acid meta</td>
<td>32.4</td>
<td>21.9</td>
<td>30.0</td>
<td>-70.6</td>
<td>51.0</td>
</tr>
</tbody>
</table>

\(\Delta G^0\), Gibbs free energy at \(T = 298.15\) K.
CONCLUSION

A new product, transient acid metarhodopsin, in the photolysis of octopus rhodopsin was detected by the time-resolved transient grating method. The lifetime at 15°C is 180 µs, which is about 5 times slower than the final absorption change. The protein structure around the chromophore should be very similar in both species, but the structures apart from the chromophore must be different. The volume change of $\Delta V = 13$ ml/mol was obtained for transient acid metarhodopsin $\rightarrow$ acid metarhodopsin transform. The activation energy for this movement is much smaller than those for the structural movement around the chromophore. Since stable acid metarhodopsin has not been shown to activate the G protein, this transient acid metarhodopsin may be responsible for G protein activation.

REFERENCES


