Structural Changes in the Lumirhodopsin-to-Metarhodopsin I Conversion of Air-Dried Bovine Rhodopsin†

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ABSTRACT: Structural changes during the photochemical reactions of unhydrated air-dried films of bovine rhodopsin in rod outer segments were examined by visible and Fourier transform infrared (FTIR) spectroscopy at 200, 240, and 280 K. These films exhibited conversion from a lumirhodopsin state to a metarhodopsin I state with a time constant of 13.5 min at 280 K, but did not form metarhodopsin II at all, as observed earlier for digitonin-extracted rhodopsin in dry gelatin films [Wald, Durell, and St. George (1950) *Science 111*, 179−181]. Lumirhodopsin which was stable in the dry film was very similar to normal lumirhodopsin. The metarhodopsin I-like state retained properties characteristic of lumirhodopsin in regard to a twisted structure between the C_{14}−H and the Schiff base of the chromophore, and perturbation around Glu122, although the C−C stretch frequencies of the chromophore were identical with those of metarhodopsin I. Thus, under dry conditions some of the structural changes that lead to metarhodopsin I are partially inhibited. These defects could result in stable lumirhodopsin and the failure to form metarhodopsin II, which is in equilibrium with metarhodopsin I.

Rhodopsin is a G protein-coupled photoreceptor in vision. This protein spans across the disk membrane in the rod outer segments (ROS)[1] of the retina. It contains 11-*cis* retinal as the chromophore linked to Lys296 through a protonated Schiff base. The light energy absorbed in the retinylidene chromophore causes isomerization to the all-trans form, yielding a series of intermediates called photorhodopsin, bathrhodopsin, lumirhodopsin (Lumi), metarhodopsin I (Meta I), and metarhodopsin II (Meta II) (Shichida, 1986). G-protein activation is carried out by Meta II (Hoffmann, 1986), so that the light information acquired by rhodopsin is transferred into the cytoplasmic enzyme cascade. It is finally transmitted to the plasma membrane and converted to an electrical signal.

The photochemical intermediates can be stabilized at low temperatures. Previous low-temperature studies applying Fourier transform infrared (FTIR) technique used hydrated ROS films. They exhibited the same photochemical reaction as in suspensions in a glycerol−water mixture and revealed their important structural properties including internal water molecules (Ganter et al., 1988a,b; Sasaki et al., 1991; Klinger & Braiman, 1992; Maeda et al., 1993; Ohkita et al., 1995).

Wald et al. (1950) have shown that dry gelatin-films of rhodopsin gave Lumi and Meta I even at room temperature and Meta II was not produced at all. The inhibition from Meta I stage was also shown for digitonin-extracted rhodopsin (Resek et al., 1993) and detergent-solubilized rhodopsin in trehalose−water glass (Sikora et al., 1994). Visible spectra of these photoproducts, however, have not been compared with standard ones which were obtained with care to avoid other photoproducts. Also, FTIR spectroscopy, which is useful for analyzing the structural changes of the protein as well as the chromophore, has not been applied to these stable intermediates.

In order to understand the mechanism of forming Meta II, it will be informative to analyze the structural characteristics of Lumi and Meta I that do not convert to Meta II. We used air-dried ROS films which also did not produce Meta II even at room temperature. Furthermore, they are free of any additional substances that disturb FTIR recording by their strong absorption, such as detergents, gelatin, and so on. Since the only difference between the hydrated and the dry films is the amount of water, the spectral differences observed between them can be ascribed to direct or indirect roles of water.

In the present study, we applied visible and FTIR spectroscopic methods to examine the structures of Lumi and Meta I of water-depleted rhodopsin and attempted to describe the states in the unhydrated ROS that cannot complete the photochemical reaction to attain Meta II.

MATERIALS AND METHODS

Dry Films. Rhodopsin in ROS of bovine retina was prepared by the method of Papermaster and Dreyer (1974) modified by Sasaki et al. (1991). Its C_{12}−^{2}H derivative was synthesized as described (Broek & Lugtenburg, 1981; Groesbeek & Lugtenburg, 1992). A 40 μL aliquot of ROS suspension (A_{500nm} = 1.0) was placed on a BaF_2 window (*d*

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[1] Abbreviations: Lumi, lumirhodopsin; Meta I, metarhodopsin I; Meta II, metarhodopsin II; ROS, rod outer segments; FTIR, Fourier transform infrared; SVD, singular value decomposition; Iso, isorhodopsin; Rho, rhodopsin; HOOP, hydrogen-out-of-plane vibration; CD, circular dichroism.

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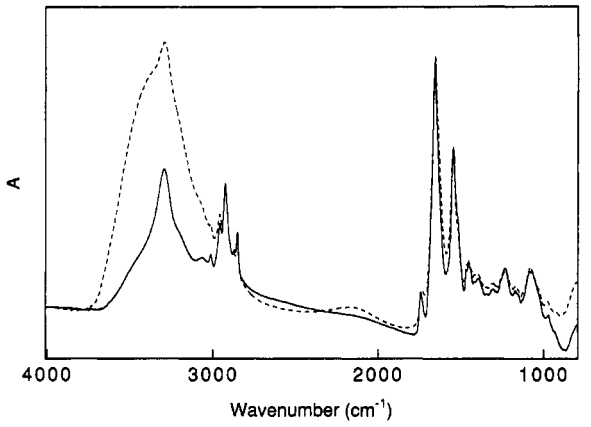

FIGURE 1: Absolute FTIR spectra of the dry film (solid line) and of the same film hydrated by 0.5 μL of water (dashed line). The full length of the ordinate is 0.7 absorbance unit.

= 10 mm) and dried in a chamber with 30% relative humidity. Without further hydration, the sample was mounted in an Oxford DN1704 cryostat under dim red light. This was denoted as the "dry film" in this paper.

The dry film exhibited a λ_{max} at 497 nm at 280 K. Its absolute FTIR spectrum is shown in Figure 1, along with that of a hydrated film. The amount of water that causes the increase in absorbance at 3500 cm^{-1} in the hydrated film can be calculated to be 42% (w/w) by using the molecular extinctions of water of 100/cm at 3500 cm^{-1} (Barbetta & Edgell, 1978) and of amide II of 150 000/cm at 1550 cm^{-1}.[2] By comparing the absorbances at 3500 cm^{-1}, the amount of water in the unhydrated film is estimated to be less than 15%.

Spectroscopic Procedures. Temperature was maintained at 200 K, 240 K, or 280 K with an Oxford ITC-4 temperature controller. Visible spectra were recorded in a Shimadzu MPS-2000 recording spectrophotometer. The whole scan from 700 to 350 nm took 52 s and was repeated every 127 s. The FTIR spectrum, recorded by adding 64 interferograms in a BioRad FTIR spectrometer FTS60A/896 at 2 cm^{-1} resolution, took 37 s. The spectrum of the light-dependent reaction was obtained as the difference between those after and before the illumination. Baseline distortion was corrected by subtracting the corresponding difference spectrum recorded for the same sample without illumination. Usually four to five recordings were conducted with fresh films. The light source for the illumination was a 1-kW halogen-tungsten lamp. The wavelength for the illumination was selected with a Toshiba filter VR58, which passes >560 nm light.

Data Analysis. Time-dependent spectra were subjected to singular value decomposition (SVD) (Henry & Hofrichter, 1992) by the use of a program, SPSERV, purchased from Dr. Csaba Bagyinka (Biological Research Center of the Hungarian Academy of Science, Szeged, Hungary). Among wavelength-dependent spectra (U spectra), weight factors (S), and illumination time-dependent vectors (V vectors), the first one or two of U spectra and V vectors were beyond noise level while the others could be regarded as random noise. If this number is two, the spectral changes consist of two components, reflecting two photochemical reactions. The two V vectors could be then fitted with two exponential

[2] This value was obtained by measuring the absorbances at 1550 cm^{-1} and 500 nm for the same air-dried film.

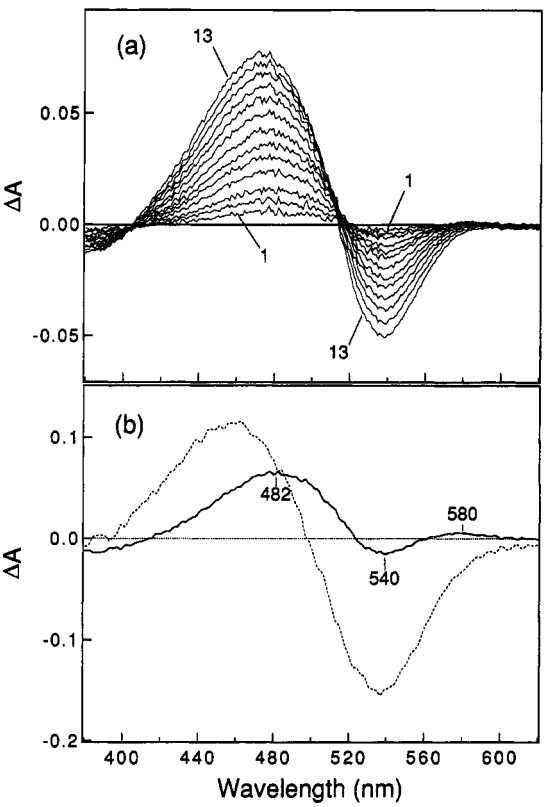

FIGURE 2: (a) A series of difference spectra upon illumination of rhodopsin in the hydrated film at 200 K. Spectra 1 and 13 are for 1 and 47 s, respectively. The other spectra between them are depicted in order of increasing duration for the illumination, 2, 3, 5, 7, 9, 12, 16, 20, 25, 31, 38 s. The absorbance at λ_{max} (501 nm) of rhodopsin was 0.83. (b) The Lumi *minus* Rho (solid line) and Iso *minus* Rho (dotted line) spectra are derived from SVD analysis of spectra in (a).

functions with τ_1 and τ_2. The difference spectra corresponding to these functions (B_1 and B_2) were calculated as follows:

$$B_1 = (U_1 \times S_1 \times a_{1,1}) + (U_s \times S_2 \times a_{2,1})$$

$$B_2 = (U_1 \times S_1 \times a_{1,2}) + (U_2 \times S_2 \times a_{2,2})$$

where $a_{1,1}$ and $a_{1,2}$ are the amplitudes of the two exponentials that fit V_1, and $a_{2,1}$ and $a_{2,2}$ are those of the same exponentials that fit V_2, respectively.

RESULTS

Standard Visible Spectral Changes upon Lumi and Meta I Formation. Standard difference spectra upon Lumi and Meta I formation have not been presented in the literature. The spectra of Lumi could be obtained by illumination of a hydrated film at 200 K with >560 nm light. The contribution of isorhodopsin (Iso), 9-*cis* retinal rhodopsin, formed by the photoreaction of Lumi (Maeda et al., 1993), was removed by the procedure described below.

A series of difference spectra upon each intermittent short illumination of hydrated film at 200 K were recorded against the spectrum of unphotolyzed rhodopsin (Rho) (Figure 2a). SVD analysis indicated that the spectral changes are derived from two photochemical reactions during the illumination. The difference spectrum due to the first photoreaction with τ_1 = 11 s (solid line in Figure 2b) was assigned to the Lumi *minus* Rho spectrum. It shows a positive band at 482 nm

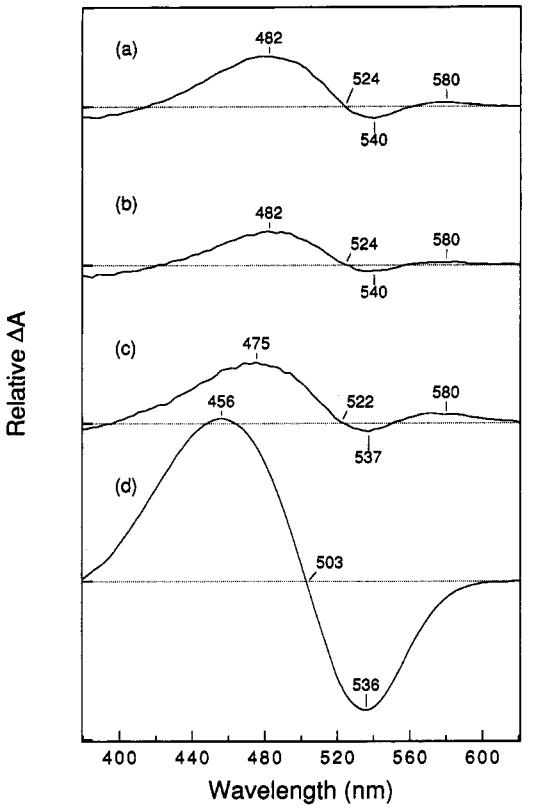

FIGURE 3: Difference visible spectra of the first photoproducts from (a) the hydrated film at 200 K, (b) the dry film at 200 K, (c) the dry film at 240 K, and (d) the hydrated film at 240 K. All of the spectra were normalized to the absorbance of Rho at λ_{max} (501 nm). Upon its adjustment to 1 unit, the full length of the ordinate is 1.13 units. All of the spectra were smoothed. The spectrum (a) is derived from the spectrum of the solid line in Figure 2b.

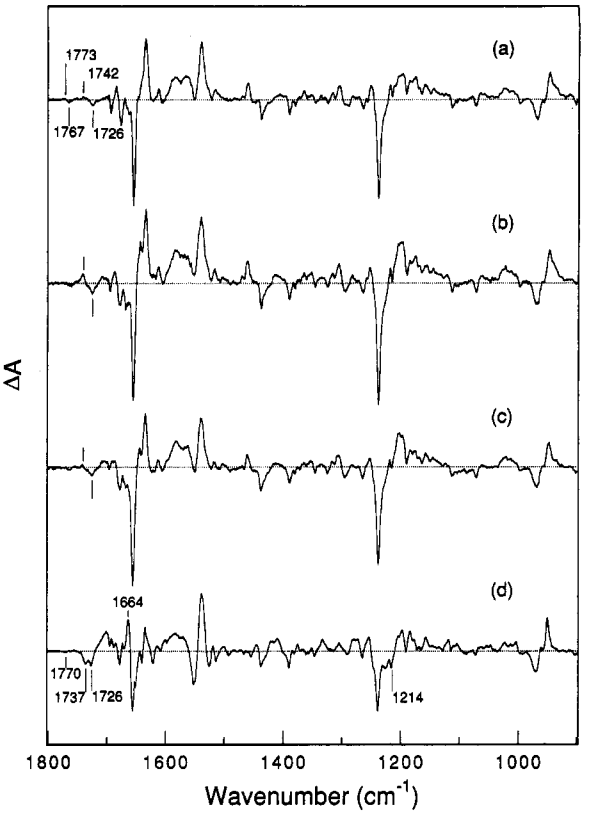

FIGURE 4: Difference FTIR spectra in the 1800−900 cm^{-1} region of (a) the hydrated film at 200 K, (b) the dry film at 200 K, (c) the dry film at 240 K, and (d) the hydrated film at 240 K. (a) and (d) are difference spectra of Lumi *minus* Rho and Meta I *minus* Rho, respectively, reproduced from Maeda et al. (1993). Horizontal dotted lines represent the baselines. Vertical solid lines show the bands with same wavenumbers as (a) or (d). The full length of the ordinate is 0.008 absorbance unit.

and small negative and positive bands at 540 and 580 nm, respectively. The blue-shifted spectrum due to the second photoreaction with τ_2 = 130 s (dotted line in Figure 2b) is the Iso *minus* Lumi spectrum.

Spectral changes of the photoreaction of the hydrated film at 240 K were recorded by the same procedure. SVD analysis again revealed two spectral components. The first spectral change (Figure 3d) is attributable to the Meta I *minus* Rho spectrum (Maeda et al., 1993). The contribution of Iso as the second photoproduct is smaller because Meta I absorbs less >560 nm light than Lumi. The Meta I *minus* Rho spectrum thus obtained crosses the baseline at a shorter wavelength, 503 nm, and forms a larger negative band at 536 nm than does the Lumi *minus* Rho spectrum. Moreover, it does not show the positive band around 580 nm.

Identity of Stable Lumi in the Dry Films. The same procedure was applied to the photoreaction of the dry film at 200 and 240 K. The difference spectrum due to the first photoreaction at 200 K (Figure 3b) coincided well with the Lumi *minus* Rho spectrum of the hydrated film at 200 K (Figure 3a; the solid line in Figure 2b after smoothing) with respect to the small negative and positive bands at 540 and 580 nm, respectively.

Slight blue shifts in the spectrum at 240 K (Figure 3c) relative to that at 200 K (Figure 3b) might be caused by temperature effects. Another study showed that the Lumi thus formed is stable during the measurements in the dark (not shown in figures). These results indicate that Lumi is more stabilized in the dry film.

We then applied FTIR spectroscopy to the photoproducts of the dry film at 200 K (Figure 4b) and 240 K (Figure 4c). The resulting spectrum was similar to the Lumi *minus* Rho spectrum (Figure 4a) and entirely different from Meta I *minus* Rho spectrum of the hydrated film at 200 K (Figure 4d), confirming that stable Lumi in the dry film is identical with Lumi in the hydrated film.

Conversion of Lumi to a Meta I State in the Dry Film at 280 K. The decay of Lumi in the dry film was observed at higher temperature. Figure 5a shows the spectral changes that occur in the dark upon short illumination (10 s) of the dry film at 280 K. The first spectrum (curve 1) immediately after the illumination, which is reproduced by a dotted line in Figure 5b, exhibits small positive and negative bands at 580 and 534 nm, respectively, and crosses with the baseline at 515 nm. These values are similar to those of the Lumi *minus* Rho spectrum at 200 K (Figure 3a), though located at slightly shorter wavelengths with a more intense positive band at 468 nm. Such a deviation could be the result of a blue shift due to the formation of the next product during the scan, and also to temperature effects.

Subsequently, spectra were recorded (curves 2−13 in Figure 5a) in the dark up to 1520 s. The spectral shift toward the shorter wavelength was observed with a different curve-intersection at 480 nm, indicating a conversion of Lumi to another state. SVD analysis provided a single spectral component with τ = 13.5 min. Its spectral shape in Figure 5b, with a positive band at 458 nm, and the intersection with

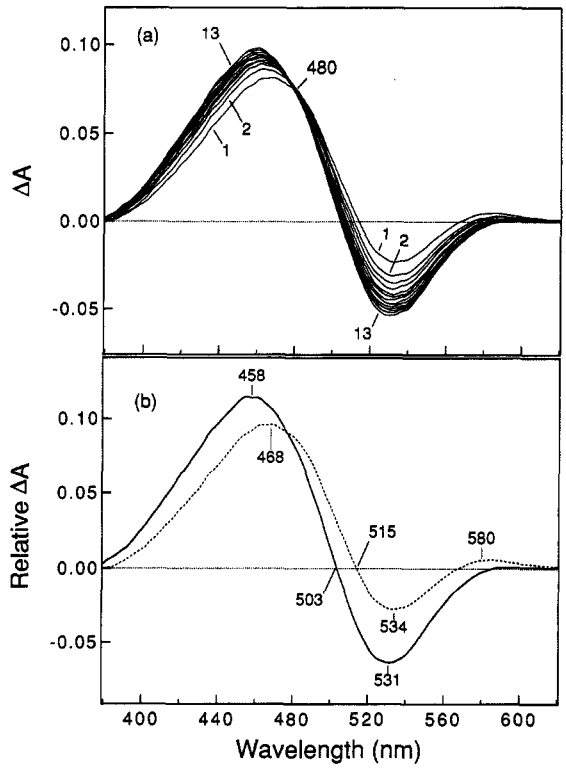

FIGURE 5: (a) Difference visible spectrum recorded immediately after illumination of rhodopsin in the dry film at 280 K for 10 s (spectrum 1) and the subsequent spectral changes up to 1524 s (spectrum 13). Meanwhile, the spectra were repeatedly recorded every 127 s. These spectra (spectra 2−13) appeared in the order of increasing time. The absorbance at λ_{max} (501 nm) of rhodopsin was 0.94. (b) Spectrum 1 reproduced from (a) (dotted line) and the calculated spectrum by fitting a single exponential function to spectra 2−13 in (a) (solid line). The spectral intensity was adjusted to the absorbance of rhodopsin at the maximum (497 nm) as 1 unit.

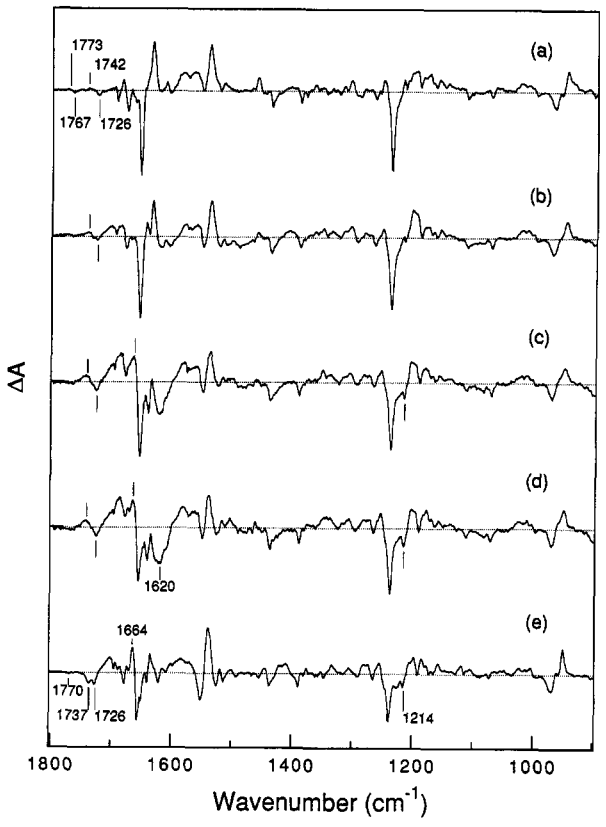

FIGURE 6: Different FTIR spectra in the 1800−900 cm^{-1} region of the dry film at 280 K after the 20 s illumination. (b) The spectrum immediately after the illumination. (c) The spectrum after 1178 s in the dark. (d) The calculated spectrum of the Meta I−like state converted from the first photoproduct. See the text for the details. (a) and (e) are difference spectra of Lumi *minus* Rho and Meta I *minus* Rho, respectively, as in Figure 4. The horizontal dotted lines represent the baselines. The vertical solid lines show the bands with the same wavenumbers as (a) or (e). The full length of the ordinate is 0.010 absorbance unit.

the baseline at 503 nm are similar to those of the Meta I *minus* Rho spectrum at 240 K (Figure 3d). Thus, in the dry film at 280 K, Lumi in a metastable state converts to Meta I with the rate constant of 13.5 min. No traces of Meta II were detected, at least within 2 h (not shown in figures).

Structure of Meta I in the Dry Film. At 280 K, where the first photoproduct decayed with τ = 13.5 min, 20 FTIR difference spectra were recorded for the dry film at every 62 s immediately after illumination for 20 s (not shown in figures). The first spectrum in the 1800−910 cm^{-1} region (Figure 6b) was similar to that of the Lumi *minus* Rho spectrum of the hydrated film at 200 K in view of the shapes of spectra in the 1250−1200 cm^{-1} and 1700−1600 cm^{-1} regions (Figure 6a).

The successive 19 spectra in the dark were subjected to SVD analysis. Because of larger noise than in the visible spectra, and baseline distortion especially in the region between 1700−1500 cm^{-1}, it was not possible to obtain an accurate τ value by fitting a V_1 vector with an exponential function. However, the fitting with τ = 13.5 min, which was obtained by visible spectroscopy, looked reasonable. Using this value, the difference spectrum of the Meta I−like state was calculated (Figure 6d). The last spectrum recorded at 1178 s was also shown for a comparison (Figure 6c). These two spectra are nearly coincident in the region below 1700 cm^{-1} except for slight distortion around 1620 cm^{-1}. A new pronounced negative band at 1214 cm^{-1} (Maeda et al., 1993), which is assigned to the C_8−C_9 stretching vibration of Rho (Palings et al., 1987; Ganter et al., 1988a), and a positive band at 1664 cm^{-1} probably due to amide I, reproduce those in the authentic Meta I *minus* Rho spectrum (Figure 6e).

The bilobe with a positive band at 1742 cm^{-1} and a negative band at 1726 cm^{-1} in the first spectrum at 280 K (Figure 6b) resembles that observed in the Lumi *minus* Rho spectrum (Figure 6a). It persists in the conversion process from Lumi to the Meta I−like state (Figures 6c, 6d) with increased intensities, but the negative band at 1737 cm^{-1} characteristic of the Meta I *minus* Rho spectrum (Ganter et al., 1989, Figure 6e) does not appear. On the basis of the assignments of the bands in the Meta II *minus* Rho spectrum (Fahmy et al., 1993), the negative band at 1737 cm^{-1} of Lumi is attributable to the carboxylic C=O of Glu122 and that at 1726 cm^{-1} tentatively to the peptide C=O of Glu122. The spectra in the process for the conversion at 280 K (Figure 6b−6d) showed neither a pair of positive and negative bands at 1773 and 1767 cm^{-1} in the Lumi *minus* Rho spectrum (Figure 6a) nor a negative band at 1770 cm^{-1} in the Meta I *minus* Rho spectrum, both of which are due to Asp83.

The C_{12}−^2H derivative of Meta I exhibits a 14-hydrogen-out-of-plane vibration (HOOP)[1] band at 886 cm^{-1} (Figure 7e), which appears neither in Lumi (Figure 7a) nor in unlabeled Meta I (Ohkita et al., 1995). The corresponding bands are observed for the C_{12}−^2H derivative of Rho and

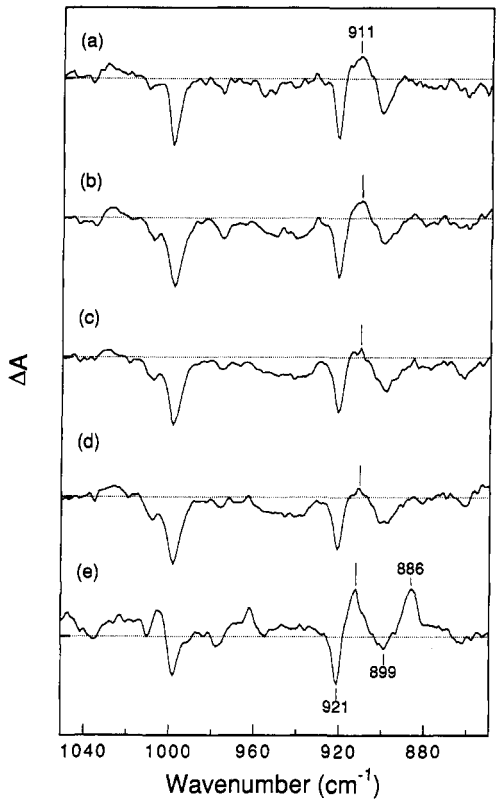

FIGURE 7: Difference FTIR spectra in the 1050−850 cm^{-1} region of the $C_{12}-^2H$ derivative of rhodopsin in the dry film at 280 K. (b) Spectra immediately after the illumination. (c) Spectra 10 min and (d) spectra 20 min after the illumination. (a) The Lumi minus Rho spectrum and (e) the Meta I minus Rho spectrum for the $C_{12}-^2H$ derivative reproduced from the previous paper (Ohkita et al., 1995). Horizontal dotted lines represent the baselines. Vertical solid lines show the bands with same wavenumbers as (a) or (e). The full length of the ordinate is 0.0052 absorbance unit.

Iso. The absence of the 14-HOOP band in Lumi in the $C_{12}-^2H$ derivative was interpreted to originate from the twist of the $C_{14}-C_{15}$ bond in the escape of the $C_{14}-H$ bond from the influence of the electrical field of the Schiff base. A neighboring band at 911 cm^{-1} is due to an isolated 11-HOOP, which forms a coupled mode with 12-HOOP at 947 cm^{-1} in the unlabeled Lumi. The intensity of the 911 cm^{-1} band of Lumi (Figure 7a) is smaller than that in Meta I (Figure 7e).

The photoreaction of the dry film of the $C_{12}-^2H$ derivative was conducted at 280 K. The positive side of the spectra in Figure 7b corresponding to Lumi did not display the band at 886 cm^{-1}. This band characteristic of Meta I did not appear at 10 min (Figure 7c) and 20 min (Figure 7d) when the reaction proceeded almost completely to the Meta I−like state. Meanwhile, the 911 cm^{-1} band rather decreased its intensity in contrast to that in Meta I, which increased its intensity (Figure 7e). The results indicate conservation of the distorted structure around the Schiff base of Lumi.

DISCUSSION

Applying visible and FTIR spectroscopic techniques, we examined the photochemical reactions and structures of Lumi and Meta I states of the air-dried ROS films. In this study we observed the effects of water on the structural changes in the conversion from Lumi to Meta I.

A stabilized Lumi state in the dry films has the same structure as in the hydrated film, as revealed by visible and FTIR spectral shape at 200 and 240 K. These confirm that Lumi is greatly stabilized in the dry state.

The more important finding is that a Meta I−like state of the dry films has at least two defects, which may inhibit complete conversion to Meta I. Lumi of the dry films is formed as a metastable state by the photoreaction at 280 K and then converts slowly to a Meta I−like state according to the visible spectra. FTIR spectroscopy, however, showed that the Meta I−like state retained some of properties characteristic of Lumi, namely twisted structure between the $C_{14}-H$ and the Schiff base of the chromophore (Ohkita et al., 1995) and the perturbation around Glu122 (Fahmy et al., 1993). The H-bonding change of the C=O of Asp83 in the normal process for the conversion to Meta I (Ganter et al., 1989) did not occur in either Lumi or Meta I. By contrast, the FTIR spectral shape in the fingerprint region, due to chromophore bands, was identical with that of Meta I. The results suggest that the Meta I−like state is stabilized by inhibition of the relaxation of the local structure around the Schiff base and the alteration of the protein structure around Glu122 and Asp83 in the process of transformation from Lumi to Meta I. Although not exhaustively studied, these could be the reasons why Lumi is stable and Meta I does not convert to Meta II in water-depleted ROS.

A Meta I−like state embedded in trehalose−water glass resembled the native Meta I by circular dichroism (CD)[1] and resonance energy transfer studies (Sikora et al., 1994). The FTIR studies reported in the present paper reveal that the Meta I−like state in the dry films is different from native Meta I. This is thus the first report to reveal an incomplete form of Meta I. The dry films used in this study are distinct from the vacuum-dried film with a main peak at 390 nm (Rafferty & Shichi, 1981; Ganter et al., 1988b).

It should be emphasized that there are water-mediated changes in the formation of Meta I, and the depletion of water leads directly or indirectly to inhibition of the formation of Meta II in equilibrium with Meta I. Preliminary results show that a water band appearing in Lumi and Meta I at 3533 cm^{-1} due to the internal water molecules (Maeda et al., 1993) is preserved even in the air-dried rhodopsin. The involvement of other water molecules will be studied in our new system with higher spectral quality (Kandori & Maeda, 1995).

Activation of G-protein occurs on the cytoplasmic surface of Meta II (Franke et al., 1992). On the other hand, the site for the interaction between the Schiff base and the protein is located closer to the intradiscal surface. A communication through the intermembrane sites around Glu122 and Asp83 may be required. The relation of these defects and water structural changes observed in the O−H stretching vibration upon conversion to Lumi and Meta I must be investigated.

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