Structural Changes in Lumirhodopsin and Metarhodopsin I Studied by Their Photoreactions at 77 K†

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ABSTRACT: The functional process of rhodopsin is initiated by cis−trans photoisomerization of the retinal chromophore. One of the primary intermediates, bathorhodopsin (Batho), is stable at 77 K, and structural changes in Batho are limited around the chromophore. Then, relaxation of Batho leads to helix opening at the cytoplasmic surface in metarhodopsin II (Meta II), which allows activation of a G protein transducin. Two intermediates, lumirhodopsin (Lumi) and metarhodopsin I (Meta I), appear between Batho and Meta II, and can be stabilized at 200 and 240 K, respectively. A photoaffinity labeling experiment reported that formation of Lumi accompanied flip-over of the β-ionone ring of the retinal chromophore so that the ring portion was attached to Ala169 of helix IV [Borhan, B., Souto, M. L., Imai, H., Shichida, Y., and Nakanishi, K. (2000) *Science 288*, 2209−2212]. According to the crystal structure of bovine rhodopsin, the distance between the labeled C3 atom of the chromophore and Ala169 was >15 Å [Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science 289*, 739−745]. These facts suggest that global protein structural changes such as helix motions take place in Lumi. In the study presented here, Lumi and Meta I are illuminated at 77 K, and protein structural changes are probed by Fourier transform infrared (FTIR) spectroscopy. We found that Lumi can be photoconverted to rhodopsin at 77 K from the IR spectral analysis of the photoproducts of Lumi. In contrast, more complex spectra were obtained for the photoproducts of Meta I at 77 K, implying that the protein structure of Meta I is considerably altered so as not to be reverted to the original state at 77 K. Thus, these photoreaction experiments with Lumi and Meta I at 77 K suggested the presence of global protein structural changes in the process between them. We concluded that the helix motions do not occur at Lumi, but at Meta I, and the flip-over of the β-ionone ring reported by the photoaffinity labeling takes place through the specific reaction channel without a change in the global structure.

Rhodopsin is one of the G protein-coupled receptors that has diverged into a photoreceptive protein in retinal visual cells (*1*−*4*). It is a membrane protein consisting of a single polypeptide opsin and a light-absorbing chromophore 11-*cis*-retinal. The opsin contains seven transmembrane α-helices, the structural motif typical of the G protein-coupled receptors. The 11-*cis*-retinal is bound to Lys296 in transmembrane helix 7 through a protonated Schiff base linkage. Absorption of a photon by the chromophore causes isomerization to the all-*trans* form, followed by conformational changes of the protein (*5*). Several intermediate states in the bleaching process are identified as photorhodopsin, bathorhodopsin (Batho),[1] lumirhodopsin (Lumi), metarhodopsin I (Meta I), and metarhodopsin II (Meta II) (*6*). Meta II catalyzes the GDP−GTP exchange reaction in the trimeric G protein transducin (*7*).

A spin labeling experiment showed that the helix opening at the cytoplasmic surface of helices III and VI when Meta II was generated (*8*). Such helix opening probably occurs by the rigid body motions of helices III and VI so that the Meta II structure is able to activate transducin. Similar results were also obtained by the introduction of a metal ion-binding site between helices III and VI (*9*). The structural changes occurring at the cytoplasmic surface are initiated by the photoisomerization of the retinal chromophore from the 11-*cis*- to the all-*trans* form (*10*). Photoisomerization takes place in the electronically excited state of rhodopsin (*11*, *12*), and photorhodopsin and Batho are formed on femto- and picosecond regimes, respectively (*5*, *6*). It should be a reasonable postulation that protein structure is not largely altered in Batho, because its formation time is in picoseconds. Batho can be stabilized at 77 K, where global structural changes are also prohibited. Very limited structural changes have indeed been proven for the K intermediate of bacterio-

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[1] Abbreviations: Batho, bathorhodopsin; Lumi, lumirhodopsin; Meta I, metarhodopsin I; Meta II, metarhodopsin II; FTIR, Fourier transform infrared; UV, ultraviolet; ROS, rod outer segments; Iso, isorhodopsin; HOOP, hydrogen-out-of-plane vibration.

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rhodopsin by means of low-temperature spectroscopic (*13*) and diffraction (*14*−*16*) studies.

How is the local structural alteration in Batho extended globally in Meta II? Two intermediates, Lumi and Meta I, appear between Batho and Meta II, and can be stabilized at 200 and 240 K, respectively. Thus, structural changes in Lumi and Meta I are important and interesting issues. Nevertheless, only limited structural information has been gained for Lumi and Meta I. Previous Fourier transform infrared (FTIR) spectroscopy studies revealed the IR spectral changes in Lumi and Meta I (*17*, *18*); on the other hand, such IR signals probe local interactions of chemical bonds, and it was not easy to correlate the signals with the global protein changes.

In 2000, Borhan et al. reported that β-ionone ring flips over at the Lumi state in a low-temperature photoaffinity labeling experiment (*19*). They introduced a photoaffinity label into the C3 atom of the β-ionone ring, and assessed the photoaffinity labeling into particular amino acids. They observed Trp265 labeled in rhodopsin and Batho, while Ala169 was labeled in Lumi, Meta I, and Meta II (*19*). On the basis of these results, they concluded that the β-ionone ring flips over in Lumi so that the ring interacts with helix IV (Ala169) in Lumi and late intermediates, differing in rhodopsin and Batho with helix VI (Trp265).

Soon after the report by Borhan et al. (*19*), the crystal structure of bovine rhodopsin was determined (*20*). According to the structure, the β-ionone ring is in van der Waals contact with Trp265, consistent with the results with the photoaffinity label for rhodopsin (Figure 1) (*19*). In contrast, Ala169 was located far from the β-ionone ring. The distance from the C3 atom of the retinal chromophore to the Cβ atom of Ala169 was 16.3 Å (Figure 1a). Figure 1b also illustrates molecular packing between retinal and Ala169 that are composed of helices III (green) and V (orange), and extracellular loop II (blue). There is only a narrow window open between them. This structure indicates that the flip-over motion of the β-ionone ring to Ala169 is only possible if the window is open by structural changes in the protein. Since Ala169 is labeled in Lumi, formation of Lumi is likely to accompany such protein structural changes that may involve motions of helices III and IV and loop E-II.

This view may be inconsistent with the previous report by Yoshizawa and Wald. They commented in their report in 1963 that Lumi could be converted to rhodopsin at 77 K by means of low-temperature UV−visible spectroscopy (*21*). Since the molecular motions are largely prohibited at 77 K, this observation suggests no global structural changes in Lumi, which seems to contradict the conclusion by Borhan et al. (*19*). Thus, protein structural changes in Lumi have to be reexamined in more detail.

In this paper, we studied protein structural changes in Lumi and Meta I by means of low-temperature FTIR spectroscopy. To examine global structural changes, we illuminated Lumi and Meta I at 77 K, and protein structural changes were probed by IR difference spectra. As a result, we found that Lumi is photoconverted to rhodopsin at 77 K. This observation is consistent with the conclusion of Yoshizawa and Wald (*21*), and provides additional experimental evidence of the protein structure. In contrast, we observed more complex spectral features for the photoproducts of Meta I at 77 K. First, only a partial amount was photoconverted from Meta I to rhodopsin at 77 K. Some of Meta I was photoinactive. Protein structural changes in the main photoproducts of Meta I at 77 K made them far different from the known species such as rhodopsin and Batho. Thus, we concluded that there are global protein structural changes between Lumi and Meta I. At the Lumi state, helix motions are not likely to occur. Together with the results by photoaffinity labeling (*19*), we infer that flip-over of the β-ionone ring takes place through the specific reaction channel without a change in the global structure of the protein.

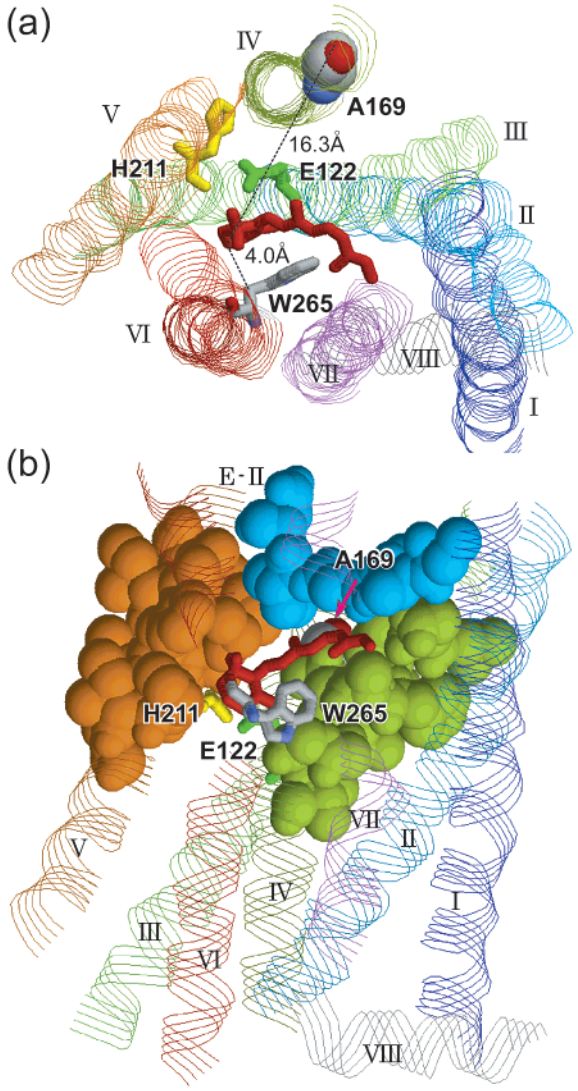

FIGURE 1: Crystal structure of bovine rhodopsin (PDB entry 1F88). (a) View from the intradiscal side. Seven transmembrane helices and helix VIII at the cytoplasmic surface are shown as ribbons. Retinal (red) attached to helix VII and Trp265 are shown as sticks, while Ala169 is shown by the CPK model. Glu122 (green) and His211 (yellow) are also shown as sticks. Distances from the C3 atom of the retinal chromophore to Cβ (nearest side chain) of Trp265 and Cβ of Ala169 are 4.0 and 16.3 Å, respectively. (b) View from the interior of the lipid bilayer. This view is made by rotating panel a 90° about a horizontal axis. Upper and lower regions correspond to the intradiscal and cytoplasmic sides, respectively. Ribbon drawings of helices VI and VII are cut away at the chromophore region for clear view. Protein moieties between the retinal chromophore and Ala169 are drawn by the CPK model: helices III (green) and V (orange), and extracellular loop II (blue). This figure shows that only a narrow window is open between retinal and Ala169, suggesting that the flip-over motion of the β-ionone ring to Ala169 needs protein structural changes that open this region.

MATERIALS AND METHODS

Frozen bovine retinas were purchased from J. A. & W. L. Lawson Co. (Lincoln, NE). Crude rod outer segments (ROS) were isolated from the retinas in ROS buffer [10 mM MOPS, 30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 0.1 mM PMSF, 1 mg/L aprotinin, 1 mg/L leupeptin, and 1 mM DTT (pH 7.3)] by a sucrose flotation method as described previously (*22*). A discontinuous sucrose gradient was used to purify ROS (*22*). ROS were then washed five times with distilled water by centrifugation at 80 000*g*. The concentration of the ROS suspension was ~10 mg/mL, which was estimated from the absorbance at 500 nm. The ROS suspension was diluted with 2 mM phosphate buffer (pH 7.0), and its final concentration was controlled to 3 mg/mL ($A_{500} = 3$).

A 90 μL aliquot of the ROS suspension was deposited on a BaF_2 window with a diameter of 18 mm and dried in the glass vessel that was evacuated by the aspirator. The film was hydrated by placing <1 μL of water next to the film. The sample was sealed by use of another window and a rubber O-ring and mounted in an Oxford DN-1704 cryostat. The experimental setup was the same as that described previously (*23*, *24*). The cryostat was equipped with an Oxford ITC-4 temperature controller, and the temperature was regulated in a range of 0.1 K. The FTIR spectra were recorded with a Bio-Rad FTS40 spectrometer at 2 cm^{-1} resolution and constructed from 128 interferograms.

In the measurement for Lumi, the illumination cycle was repeated as follows. (i) The rhodopsin sample was cooled to 77 K and irradiated with 501 nm light for 2 min and then with >610 nm light for 2 min. Irradiation with 501 nm light at 77 K converts rhodopsin to Batho (*23*). Irradiation with >610 nm light for 2 min converts Batho to rhodopsin and a small amount of isorhodopsin (Iso) (9-*cis*-rhodopsin) (*23*). This procedure (pre-illumination) was necessary to control the amount of Iso contained in the sample. (ii) The sample was warmed to 200 K and illuminated with >560 nm light for 1 min, which leads to conversion of rhodopsin to Lumi (*25*). Under these illumination conditions, formation of Lumi is almost saturated, as is seen in the appearance of the Iso-characteristic bands at 960 and 1206 cm^{-1} (*25*). (iii) The sample was cooled to 77 K and irradiated with 501 nm light for 5 min and with >610 nm light for 5 min. Then, procedures ii and iii were repeated. In the measurement for Meta I, a similar illumination cycle of procedures ii and iii for Lumi was repeated except for temperature. In procedure ii, the sample was illuminated at 240 K. In addition, pre-illumination was not applied in measuring the Meta I minus rhodopsin spectrum. The light source for the illumination was a 1 kW halogen−tungsten lamp in a slide projector.

RESULTS

Photoconversion of Lumirhodopsin at 77 K. Figure 2a shows the Lumi minus rhodopsin spectrum measured at 200 K, which reproduced the spectrum reported previously (*25*). We confirmed that formation of Lumi was almost saturated under the illumination conditions described herein. In fact, the spectrum shows the appearance of an Iso-specific 960 cm^{-1} band (Figure 2a), which originates from the photoreaction of Lumi at 200 K (*25*). Then, the sample was cooled to 77 K and illuminated first with 501 nm light for 2 min and then with >610 nm light for 2 min. After this procedure, the sample was warmed and illuminated at 200 K (Figure 2b). Difference IR spectra were obtained repeatedly at 200 K in the same way (Figure 2c,d). Figure 2e shows that the four spectra that are obtained are identical in both shape and amplitude. This result indicates that Lumi completely reverted to rhodopsin as a result of the illumination at 77 K. In this case, there are two possibilities for formation of rhodopsin. One is that rhodopsin is directly formed by illumination of Lumi at 77 K. The other is that the photoproduct of Lumi at 77 K is different from rhodopsin, and it decays to rhodopsin upon being warmed to 200 K. We are able to decide the alternatives by comparing the spectrum upon illumination of Lumi at 77 K.

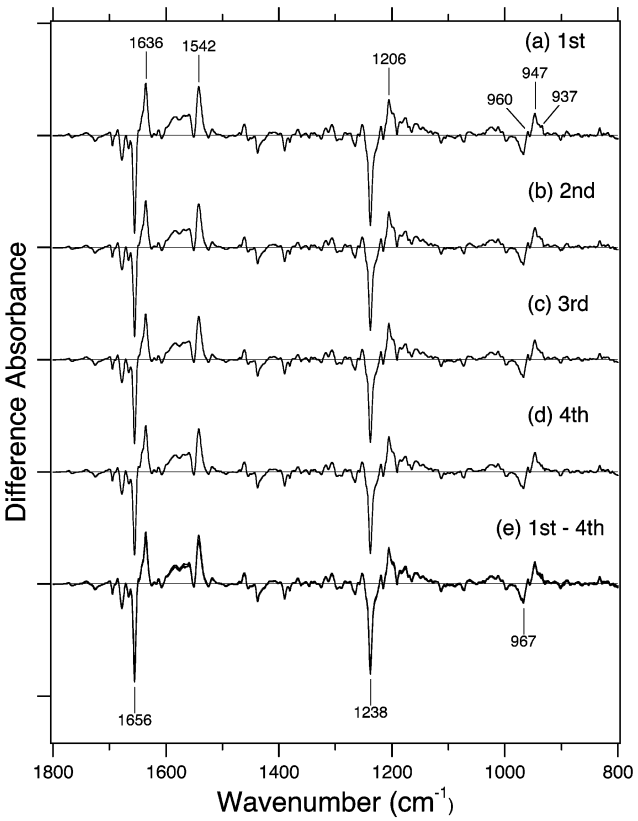

FIGURE 2: Lumi minus rhodopsin difference IR spectra measured at 200 K. After the first conversion of rhodopsin to Lumi via light (a), the sample was cooled and illuminated at 77 K to form rhodopsin, followed by illumination again at 200 K (b). This cycle was repeated, and the four spectra obtained at 200 K were superimposed in panel e. Tags in panels a and e are characteristic bands of Lumi and rhodopsin, respectively.

Figure 3a shows the difference IR spectrum recorded after minus before illumination of Lumi with 501 nm for 2 min at 77 K. Characteristic HOOP bands of Batho (921 and 851 cm^{-1}) (*26*) and Lumi (948 and 938 cm^{-1}) (*27*) appeared in the positive and negative sides, respectively. This result suggests that Lumi was converted to Batho presumably through rhodopsin (or a rhodopsin-like product) by the continuous illumination at 77 K. The presence of the 960 cm^{-1} band indicates that Iso contained in the sample is also photoconverted by illumination with 501 nm light. Figure 3b shows the Batho minus rhodopsin spectrum, which is identical to that reported previously (*23*). We then attempted to subtract the spectrum of Figure 3a from that of Figure 3b to cancel the Batho-specific 921 and 851 cm^{-1} bands. The spectrum that is obtained (Figure 3c, solid line) looks

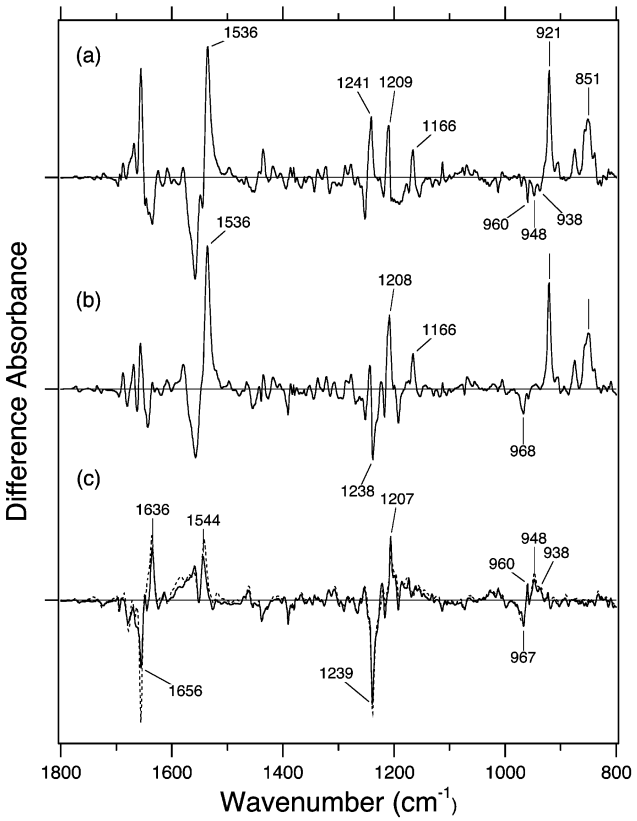

FIGURE 3: (a) Difference IR spectrum measured at 77 K. The illumination product rhodopsin at 200 K that predominantly contains Lumi was cooled at 77 K, followed by illumination with 501 nm light for 2 min. The appearance of the Batho-characteristic HOOP bands at 921 and 851 cm^{-1} indicates formation of Batho. (b) Batho minus rhodopsin difference IR spectrum measured at 77 K. (c) The solid line represents the subtraction of spectrum a from spectrum b wihch cancels the Batho-characteristic HOOP bands at 921 and 851 cm^{-1}. The dotted line represents the Lumi minus rhodopsin spectrum at 200 K reproduced from Figure 1a.

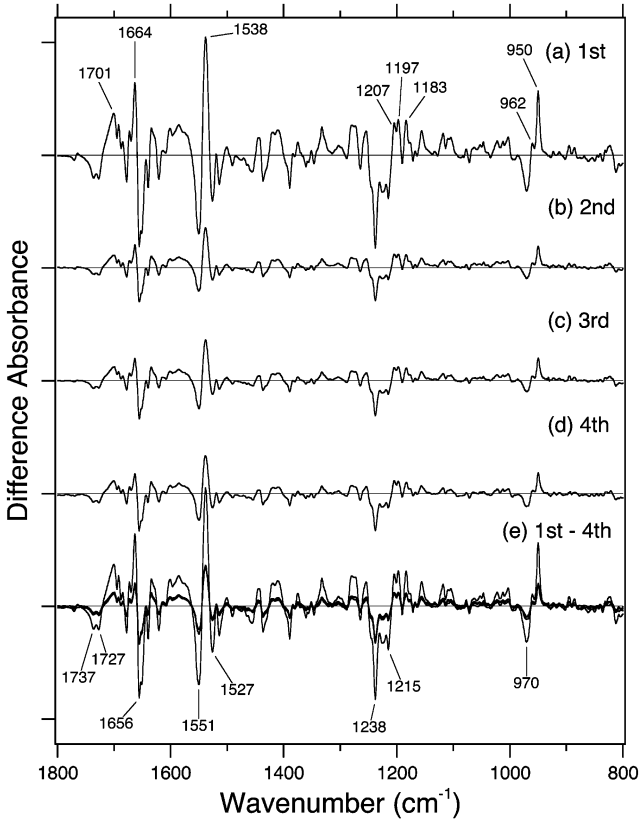

FIGURE 4: Meta I minus rhodopsin difference IR spectra measured at 240 K. After the first conversion of rhodopsin to Meta I via light (a), the sample was cooled and illuminated at 77 K to form rhodopsin, followed by illumination again at 240 K (b). This cycle was repeated, and the four spectra obtained at 240 K were superimposed in panel e. Tags in panels a and e are characteristic bands of Meta I and rhodopsin, respectively.

essentially identical to the Lumi minus rhodopsin spectrum measured at 200 K (Figure 3c, dotted line reproduced from Figure 2a). This fact implies that the photoproduct of Lumi with illumination with 501 nm light for 2 min at 77 K (Figure 3a) is Batho. Therefore, successive illumination at >610 nm converted Batho to rhodopsin, which allowed complete reversion of Lumi to rhodopsin at 77 K. Figure 3c also suggests that both rhodopsin and Lumi have similar structures between 77 and 200 K. The change in amplitude at 1656 and 1636 cm^{-1} may originate from the temperature effect. Since global protein structural changes are prohibited at 77 K, this observation suggests that formation of Lumi does not accompany such protein motion.

Photoconversion of Metarhodopsin I at 77 K. We next tested for Meta I. Figure 4a shows the Meta I minus rhodopsin spectrum measured at 240 K, which is identical to those reported previously (*25*). We confirmed that formation of Meta I is almost saturated under the illumination conditions described herein. In fact, the spectrum shows the appearance of Iso-specific bands at 962 and 1207 cm^{-1} (Figure 4a), which originates from the photoreaction of Meta I at 240 K (*25*). Then, the sample was cooled to 77 K and illuminated first with 501 nm light for 2 min and then with >610 nm light for 2 min. After this procedure, the sample was warmed and illuminated at 240 K (Figure 4b). Difference IR spectra were obtained repeatedly at 240 K in the same way (Figure 4c,d). In contrast to the case for Lumi (Figure 2), the difference spectra were reduced in amplitude from the second one (Figure 4b), whereas the spectral shapes were identical (Figure 4e). The amplitude of the spectra in panels b−d of Figure 3 was 33% of that of the first spectrum (Figure 4a), and further cycles yielded the same amplitude (data not shown). The identical spectral shape in Figure 4 indicates that Meta I can also revert to rhodopsin by means of the illumination at 77 K like Lumi, whereas the reversion is partial unlike that of Lumi. Partial reversion of Meta I strongly suggests the structural heterogeneity in Meta I.

Why does Meta I partially revert to rhodopsin? To further examine the mechanism, we applied spectral comparison by illumination of Meta I with 501 nm light for 2 min at 77 K. Solid and dotted lines in Figure 5 represent difference IR spectra after the measurement of the spectra in panels a and d of Figure 4 at 240 K, respectively. Two spectra were almost identical with each other. Identical spectra were also obtained after the measurements of the spectra in panels b and c of Figure 4 (not shown).

Spectra in Figure 5 show the characteristic HOOP bands of Batho (921 and 851 cm^{-1}) (*26*) and Meta I (950 cm^{-1}) (*27*) in the positive and negative side, respectively. This result suggests that some amount of Meta I was converted to Batho presumably through rhodopsin (or a rhodopsin-like product) by continuous illumination at 77 K. Absorbances of the 950 cm^{-1} band in Figures 4a and 5 are 0.00579 and −0.00429,

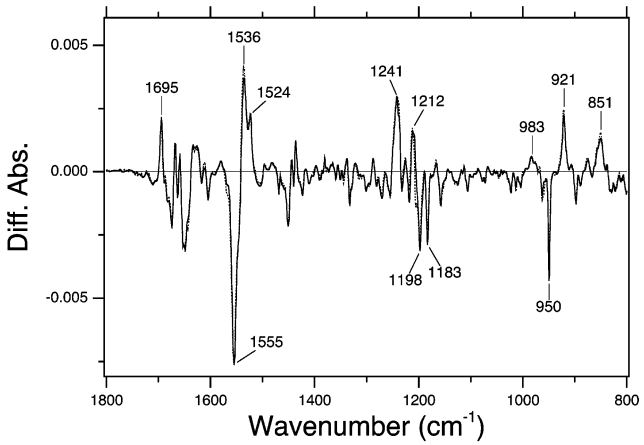

FIGURE 5: Difference spectra by illumination of Meta I with 501 nm light at 77 K. Solid and dotted lines represent difference IR spectra after the measurements of Figure 4a and Figure 4d at 240 K, respectively. The two spectra were almost identical with each other.

respectively. Therefore, most of Meta I (74%) is photoconverted to some products at 77 K. The rest of Meta I (26%) that seems inactive upon illumination may be explained by a photoequilibrium between Meta I and the photoproduct at 77 K under the illumination conditions described herein. Another possibility is that the formed product possesses the HOOP band at 950 cm^{-1}.

Figure 5 shows the appearance of the Batho-characteristic HOOP bands at 921 and 851 cm^{-1}; however, the intensities were small. It is clear if we compare the spectral feature of these bands between Figure 5 (Batho from Meta I) and Figure 3a (Batho from Lumi). It is possible to calculate the Batho minus Meta I spectrum from the Batho minus rhodopsin spectrum (Figure 3b) and the Meta I minus rhodopsin spectrum (Figure 4a) by canceling the rhodopsin-specific bands. The calculation showed that in the Batho minus Meta I spectrum, the 921 cm^{-1} band of Batho was 3.16 times greater in amplitude than the 950 cm^{-1} band of Meta I (data not shown). Since the absorbances at 950 and 921 cm^{-1} were −0.00429 and 0.00225, respectively, in Figure 5, we estimated that 17% of the photoreacted Meta I was converted to Batho. In addition to the 921 and 851 cm^{-1} bands, the positive 1536 cm^{-1} band is characteristic of Batho that corresponds to the C═C stretching vibration of the retinal chromophore (Figure 3b). Other positive bands such as those at 1695, 1524, 1241, 1212, and 983 cm^{-1} probably originate from the 83% photoproduct of Meta I. Interestingly, the positive bands at 1241 and 1212 cm^{-1} in the fingerprint region are close in frequency to the rhodopsin-specific bands at 1238 and 1215 cm^{-1}, respectively, in the Meta I minus rhodopsin spectrum (Figure 4a). This fact may suggest that illumination of Meta I at 77 K results in formation of the 11-*cis* chromophore, whereas it could not be further converted to Batho from the product. More detailed discussion requires assignment of these vibrational bands by use of isotope-labeled retinal.

This analysis suggests that upon illumination of Meta I with 501 nm light at 77 K, (i) 13% (0.17 × 0.74) was converted to Batho, (ii) 61% (0.83 × 0.74) was converted to a product (products) possibly having the 11-*cis* chromophore, and (iii) 26% was Meta I. Thus, unlike the case of Lumi, successive illumination at >610 nm does not result

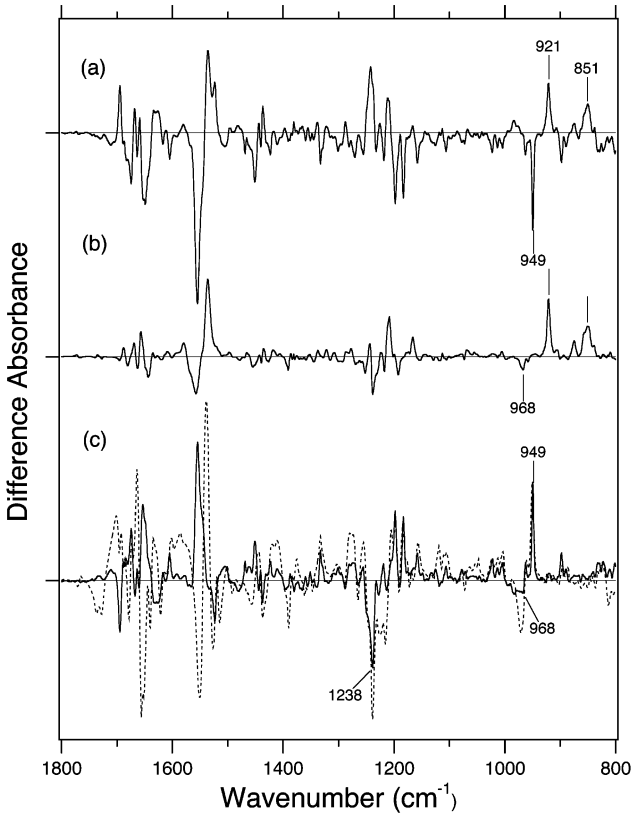

FIGURE 6: (a) Difference spectra by illumination of Meta I with 501 nm light at 77 K. Tags are characteristic HOOP bands of Batho (851 and 921 cm^{-1}) and Lumi (938 and 948 cm^{-1}). (b) Batho minus rhodopsin spectrum. Tags are characteristic HOOP bands of Batho (851 and 921 cm^{-1}) and rhodopsin (968 cm^{-1}). (c) The solid line represents the subtraction of spectrum a from spectrum b which cancels the Batho-characteristic HOOP bands at 921 and 851 cm^{-1}. The dotted line represents the Meta I minus rhodopsin spectrum at 240 K reproduced from Figure 4a.

in complete reversion to rhodopsin. When the sample was warmed to 240 K, 33% of Meta I was consequently reverted to rhodopsin. Thus, these results showed the multiple photoreaction pathways of Meta I.

Figure 6 shows the same attempt for Meta I as that for Lumi (Figure 2). Panels a and b of Figure 6 are reproduced from the solid lines in Figure 5 and Figure 3b, respectively. The photoproduct of Lumi at 77 K was Batho, so that subtraction of the photoproduct minus Lumi spectrum (Figure 3a) from the Batho minus rhodopsin spectrum (Figure 3b) coincides with the Lumi minus rhodopsin spectrum. In the case of Meta I, there is a photoproduct (photoproducts) other than Batho. So subtraction of the spectrum of Figure 6a from that of Figure 6b to cancel the Batho-specific 921 and 851 cm^{-1} bands should correspond to the Meta I minus [the photoproduct(s) + rhodopsin] spectrum, where the content of rhodopsin is minor (17%). As is clearly shown in Figure 6c, the calculated spectrum (solid line) was far different from the Meta I minus rhodopsin spectrum (dotted line). The presence of the negative 1238 cm^{-1} band may suggest that the photoproduct possesses 11-*cis*-retinal as the chromophore. It is noted that there is little spectral change in the 1750−1700 cm^{-1} region (solid line), which are the characteristic frequencies of the C═O stretch of protonated carboxylic acids. The bands at 1737 (−), 1727 (−), and 1701 (+) cm^{-1} in the Meta I minus rhodopsin spectrum (Figure 4) have been

assigned to the C═O stretch of Glu122 (*28*). Small spectral changes imply that the environment of Glu122 is persistent upon photoreaction of Meta I at 77 K.

DISCUSSION

Protein Structure in Lumi. In this study, we illuminated Lumi and Meta I at 77 K, and protein structural changes were probed by IR spectra. This work is based on the hypothesis that only local structural changes are possible at 77 K (*5*). At 77 K, protein structure is probably frozen, and photoisomerization of the retinal chromophore yields local structural alterations. Spectroscopic (*13*) and diffraction (*14*−*16*) studies on the primary K intermediate of bacteriorhodopsin indeed showed the limited structural changes around the retinal chromophore. Therefore, if Lumi and Meta I possess protein structures different from that of rhodopsin, we expected that the different photoproducts are formed by illumination at 77 K. This is indeed the case for Meta I. In contrast, Lumi was reverted to rhodopsin at 77 K (Figures 2 and 3). These results are consistent with those from low-temperature UV−visible spectroscopy (*21*, *29*), and provided additional evidence of protein structure. Not only visible absorption but also protein structure of Lumi was reverted to that of rhodopsin by illumination at 77 K.[2]

Borhan et al. (*19*) showed that the photoaffinity label in the C3 atom of the β-ionone ring is attached to Ala169 in Lumi, both of which are >15 Å from each other (Figure 1). The distance is not changed in the refined structures (*30*, *31*). Figure 1b shows a very narrow window between retinal and Ala169, and three domains, helices III and V and loop E-II, divide them. Attachment of the label to Ala169 requires opening of the domain, which should accompany motions of the helices and the loop. One possibility is that after such motion at 200 K, cooling of Lumi from 200 to 77 K displaces the retinal position from Ala169. This is unlikely because of the spectral coincidence in Figure 3c. IR bands are highly sensitive to their molecular environments, so rhodopsin and Lumi have unique structures at both 77 and 200 K. It is well-known that photoisomerization takes place with high efficiency even at very low temperatures in rhodopsin (*5*). This fact indicates the presence of the specific reaction mechanism in the protein for formation of Batho. We infer the presence of a similar mechanism for formation of Lumi, which undergoes flip-over of the β-ionone ring through the specific reaction channel without a change in the global structure. Several lines of evidence indicated that the changes in the chromophore−opsin interaction near the β-ionone ring of the chromophore could occur during the batho-to-lumi transition (*32*−*35*). These facts also support the presence of the specific reaction channel in the rhodopsin molecule.

Protein Structure in Meta I. Unlike the case in Lumi, illumination of Meta I at 77 K did not result in the conversion of Meta I to rhodopsin. As a consequence, the magnitude of the difference spectra was 33% of that of the first one (Figure 4). Nevertheless, the spectral shape was identical to that of the Meta I minus rhodopsin spectrum, indicating that Meta I could partially revert to rhodopsin. Partial reversion of Meta I strongly suggests structural heterogeneity in Meta I. From the analysis of the HOOP bands in Figure 5, it was suggested that 74% of the formed Meta I at 240 K was photoconverted to some products at 77 K. The analysis of the HOOP bands further suggested that 13% of Meta I was photoconverted to Batho, while 61% of Meta I was converted to the photoproduct(s) other than rhodopsin, Iso, and Batho. The IR bands at 1695, 1524, 1241, 1212, and 983 cm^{-1} are characteristic of the photoproduct(s). The positive bands at 1241 and 1212 cm^{-1} in the fingerprint region are close in frequency to those at 1238 and 1215 cm^{-1}, respectively, in the Meta I minus rhodopsin spectrum (Figure 4a), suggesting that the photoproduct(s) possesses an 11-*cis* chromophore.

With regard to the structure of the photoproduct(s) of Meta I at 77 K, the frequency region at 1750−1700 cm^{-1} provided some information. The Meta I minus rhodopsin spectrum possesses bands at 1737 (−), 1727 (−), and 1701 (+) cm^{-1} (Figure 4) that are much stronger than those of the Lumi minus rhodopsin spectrum (Figure 2). They have been assigned as the C═O stretch of Glu122 (*28*), which is in contact with the β-ionone ring (Figure 1). A large change in this band in Meta I is consistent with the observation by the photoaffinity labeling; namely, the environment of Glu122 was largely changed in Meta I because of the removal of the ring. If the β-ionone ring moves to the portion of Ala169 in Meta I, small spectral changes of the bands upon illumination of Meta I at 77 K (Figure 6) imply that the ring does not move back to the position in rhodopsin.

The frequency of the C═O stretch of Glu122 in Meta I (1701 cm^{-1}) is very low as the carboxylic C═O stretch, indicating a very strong hydrogen bonding interaction. According to the rhodopsin structure (*20*), the distance between an oxygen and a nitrogen of side chains of Glu122 and His211, respectively, is 3.2 Å. Therefore, upward movement of the β-ionone ring in Figure 1b toward Ala169 may yield a tight interaction of Glu122 with His211. In fact, it was reported that the C═O stretch of Glu122 in the Meta I minus rhodopsin spectrum is strongly influenced by the mutation of His211 (*28*).

These results showed the presence of global protein structural changes between Lumi and Meta I. This conclusion is clearly deduced from the spectra in Figures 3c (coincident for Lumi) and 6c (different for Meta I). A major photoproduct of Meta I (61%) at 77 K is likely to possess 11-*cis*-retinal- and Meta I-like protein structure. It is, however, noted that we also observed the 13% photoproduct exhibiting the Batho-specific HOOP band (Figure 6a). We cannot conclude that this photoproduct corresponds to Batho because of other spectral components. If Batho is indeed formed from Meta I at 77 K, this hypothesis may be questioned. The specific reaction channel of the β-ionone ring to Ala169 in Lumi may be partly open even in Meta I. It may suggest heterogeneity in the protein structure of Meta I. We need clarify this question in the future. In any case, different photoreaction pathways at 77 K are revealed between Lumi and Meta I.

CONCLUSION

On the basis of the hypothesis that global conformation changes are prohibited at 77 K, we investigated the photo-

[2] These results with Lumi are far different from those of the L intermediate of bacteriorhodopsin, though both are stabilized at similar temperatures. It is known that the L intermediate never reverts to the original bacteriorhodopsin upon illumination at 77 K (*36*), suggesting intrinsic properties of visual and archaeal rhodopsins.

reaction properties of Lumi and Meta I by means of FTIR spectroscopy. As a result, we found the significant difference between Lumi and Meta I. Low-temperature FTIR spectroscopy revealed that the photoproducts of Lumi at 77 K are constituted by those of rhodopsin and Batho, indicating that Lumi can be photoconverted to rhodopsin at this temperature. In contrast, more complex spectra were obtained for the photoproducts of Meta I at 77 K, implying that the protein structure of Meta I is considerably altered so it does not revert to the original state at 77 K. We concluded that global protein motions do not occur at Lumi, but at Meta I. We concluded that the flip-over of the β-ionone ring reported by the photoaffinity labeling takes place through the specific reaction channel without a change in the global structure. It is noted that certain structural changes take place at the β-ionone ring in Lumi, as was studied by ring-modified retinal analogues (*34*). Thus, further experimental efforts will lead to a better understanding of the structural changes in Lumi and Meta I.

REFERENCES

1. Khorana, H. G. (1992) *J. Biol. Chem. 267*, 1−4.
2. Hofmann, K.-P., and Helmreich, E. J. M. (1996) *Biochim. Biophys. Acta 1286*, 285−322.
3. Shichida, Y., and Imai, H. (1998) *Cell. Mol. Life Sci. 54*, 1299−1315.
4. Sakmar, T. P. (1998) *Prog. Nucleic Acid Res. Mol. Biol. 59*, 1−33.
5. Kandori, H., Shichida, Y., and Yoshizawa, T. (2001) *Biochemistry (Moscow) 66*, 1197−1209.
6. Shichida, Y. (1986) *Photobiochem. Photobiophys. 13*, 287−307.
7. Hofmann, K. P. (1986) *Photobiochem. Photobiophys. 13*, 309−327.
8. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) *Science 274*, 768−770.
9. Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) *Nature 383*, 347−350.
10. Kandori, H., Matuoka, S., Shichida, Y., Yoshizawa, T., Ito, M., Tsukida, K., Balogh-Nair, V., and Nakanishi, K. (1989) *Biochemistry 28*, 6460−6467.
11. Chosrowjan, H., Mataga, N., Shibata, Y., Tachibanaki, S., Kandori, H., Shichida, Y., Okada, T., and Kouyama, T. (1998) *J. Am. Chem. Soc. 120*, 9706−9707.
12. Kandori, H., Furutani, Y., Nishimura, S., Shichida, Y., Chosrowjan, H., Shibata, Y., and Mataga, N. (2001) *Chem. Phys. Lett. 334*, 271−276.
13. Kandori, H., Kinoshita, N., Yamazaki, Y., Maeda, A., Shichida, Y., Needleman, R., Lanyi, J. K., Bizounok, M., Herzfeld, J., Raap, J., and Lugtenburg, J. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 4643−4648.
14. Edman, K., Nollert, P., Royant, A., Belrhali, H., Pebay-Peyroula, E., Hajdu, J., Neutze, R., and Landau, E. M. (1999) *Nature 401*, 822−826.
15. Schobert, B., Cupp-Vickery, J., Hornak, V., Smith, S., and Lanyi, J. K. (2002) *J. Mol. Biol. 321*, 715−726.
16. Matsui, Y., Sakai, K., Murakami, M., Shiro, Y., Adachi, S., Okumura, H., and Kouyama, T. (2002) *J. Mol. Biol. 324*, 469−481.
17. Siebert, F. (1995) *Isr. J. Chem. 35*, 309−323.
18. Maeda, A., Kandori, H., Yamazaki, Y., Nishimura, S., Hatanaka, M., Chon, Y.-S., Sasaki, J., Needleman, R., and Lanyi, J. K. (1997) *J. Biochem. 121*, 399−406.
19. Borhan, B., Souto, M. L., Imai, H., Shichida, Y., and Nakanishi, K. (2000) *Science 288*, 2209−2212.
20. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science 289*, 739−745.
21. Yoshizawa, T., and Wald, G. (1963) *Nature 197*, 1279−1286.
22. Kandori, H., Matuoka, S., Shichida, Y., and Yoshizawa, T. (1989) *Photochem. Photobiol. 49*, 181−184.
23. Kandori, H., and Maeda, A. (1995) *Biochemistry 34*, 14220−14229.
24. Nishimura, S., Kandori, H., and Maeda, A. (1997) *Photochem. Photobiol. 66*, 796−801.
25. Maeda, A., Ohkita, Y. J., Sasaki, J., Shichida, Y., and Yoshizawa, T. (1993) *Biochemistry 32*, 12033−12038.
26. Bagley, K. A., Balogh-Nair, V., Croteau, A. A., Dollinger, G., Ebrey, T. G., Eisenstein, L., Hong, M. K., Nakanishi, K., and Vittitow, J. (1985) *Biochemistry 24*, 6055−6071.
27. Ganter, U. M., Gartner, W., and Siebert, F. (1988) *Biochemistry 27*, 7480−7488.
28. Beck, M., Sakmar, T. P., and Siebert, F. (1998) *Biochemistry 37*, 7630−7639.
29. Becher, B. (1980) *Biophys. J. 30*, 1−7.
30. Teller, D. C., Okada, T., Behnke, C. A., Palczewski, K., and Stenkamp, R. E. (2001) *Biochemistry 40*, 7761−7772.
31. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 5982−5987.
32. Hug, S. J., Lewis, J. W., Einterz, C. M., Thorgeirsson, T. E., and Kliger, D. S. (1990) *Biochemistry 29*, 1475−1485.
33. Maeda, A., Shichida, Y., and Yoshizawa, T. (1979) *Biochemistry 18*, 1449−1453.
34. Okada, T., Kandori, H., Shichida, Y., Yoshizawa, T., Denny, M., Zhang, B. W., Asato, A. E., and Liu, R. S. H. (1991) *Biochemistry 30*, 4796−4802.
35. Shichida, Y., Tokunaga, F., and Yoshizawa, T. (1978) *Biochim. Biophys. Acta 504*, 413−430.
36. Litvin, F. F., and Balashov, S. P. (1977) *Biophysics (Engl. Transl.) 22*, 1157−1160.

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