Volume 280, number 1, 107–111 FEBS 09470 March 1991

ADONIS 001457939100180Z

Fourier transform infrared spectroscopic study on retinochrome and its primary photoproduct, lumiretinochrome

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Received 3 January 1991

Structural studies of retinochrome, and its photoproduct, lumiretinochrome, were done by Fourier transform infrared difference spectroscopy. The absorption bands in the carbonyl stretching region which shift in D_2O show the changes in the protein part during the photoreaction. Strong absorption bands in the finger-print region show that the all-*trans*-retinal chromophore in retinochrome isomerizes to the 11-*cis*-retinal chromophore in lumiretinochrome upon illumination with yellow-green light at 83K.

Retinochrome; Lumiretinochrome; Fourier transform infrared spectroscopy

1. INTRODUCTION

Retinochrome is a photosensitive protein existing in the visual cells of cephalopods [1]. In contrast to the other photosensitive protein, cephalopod rhodopsin, which has an 11 *cis*-retinal chromophore bound to a lysine residue and is located in the rhabdomal membranes of the outer segments, retinochrome has an all-*trans*-retinal and is located in the inner portions of the cells. The all-*trans*-chromophore of retinochrome is isomerized to the 11-*cis*-form during photoreaction [2]. This information together with the existence of the retinal binding protein which can transport retinal between retinochrome and rhodopsin indicate that the function of retinochrome is the regeneration of 11-*cis*-retinal from all-*trans*-retinal produced by rhodopsin photoreaction [3].

Much attention has been focused on the characterization of the photointermediates of bovine rhodopsin (Rh) and bacteriorhodopsin (bR). Photo-excitation and relaxation processes of Rh and bR have been studied using a variety of spectroscopic methods and the structures of both the chromophore and proteins of the various intermediates produced during photoreaction have been discussed in detail [4]. Photo-reaction of retinochrome has been studied mainly by UV-visible absorption spectrophotometry. In the low temperature studies of the photolytic process of retinochrome [2,5] (λ_{max} 496 nm), the intermediates, prelumiretinochrome (λ_{max} 465 nm), lumiretinochrome (λ_{max} 475 nm), and metaretinochrome (λ_{max} 470 nm) were trapped at 25K, 83K and 273K, respectively. The structure of retinochrome intermediates has been identified only with chromophore extraction [2] and further information on the structure has not been presented. Here we report structural information on retinochrome using a Fourier transform infrared (FTIR) difference spectroscopic study.

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Abbreviations: FTIR, Fourier transform infrared spectroscopy; Rh, bovine rhodopsin; bR, bacteriorhodopsin; Rc, retinochrome.

2. MATERIALS AND METHODS

All chemicals were purchased from Nacalai Tesque (Kyoto). All-*trans*-retinal-15-*d* was prepared as follows: methylation of commercial all-*trans*-retinoic acid with CH_2N_2 in ether, reduction of the resulting ester with $LiAlD_4$ to retinol-15-d_2 and MnO_2 oxidation of the retinol in hexane. All-*trans*-retinal-15-*d* and commercial all-*trans*-retinal were purified by HPLC (column: Cosmosil Si-60, ID 10 mm × 300 mm, solvent: 10 % ethyl ether in hexane, flow rate: 6 ml/min).

Suspensions of retinochrome in the membrane and apomembrane were prepared from the retina of the squid, *Todarodes pacificus* [6]. Retinochrome samples were also regenerated by the incorporation of all-*trans*-retinal and all-*trans*-retinal-15-d_1 (C_{15}-*d*) into bleached apomembranes. The membrane suspensions were centrifuged and the pellets were suspended in H_2O for FTIR measurement. Proton/deuteron (H/D) exchange of exchangeable hydrogen was performed by repeated lyophilization followed by addition of D_2O. The suspension was dried up to film on a ZnS window of a sample holder and hydrated by the addition of 10 μl of water, followed by incubation in a container with 100% humidity for one hour, then it was sealed with a Ge window. The concentration of the samples was adjusted to be about 0.1 OD at 500 nm. It was then installed into an Oxford CF1204 type cryostat equipped with ZnS windows which was set up in the sample compartment of a Nicolet 7199 series FTIR spectrometer. Lumiretinochrome (lumiRc) and metaretinochrome (metaRc) were prepared by illumination with 250 Watt optical

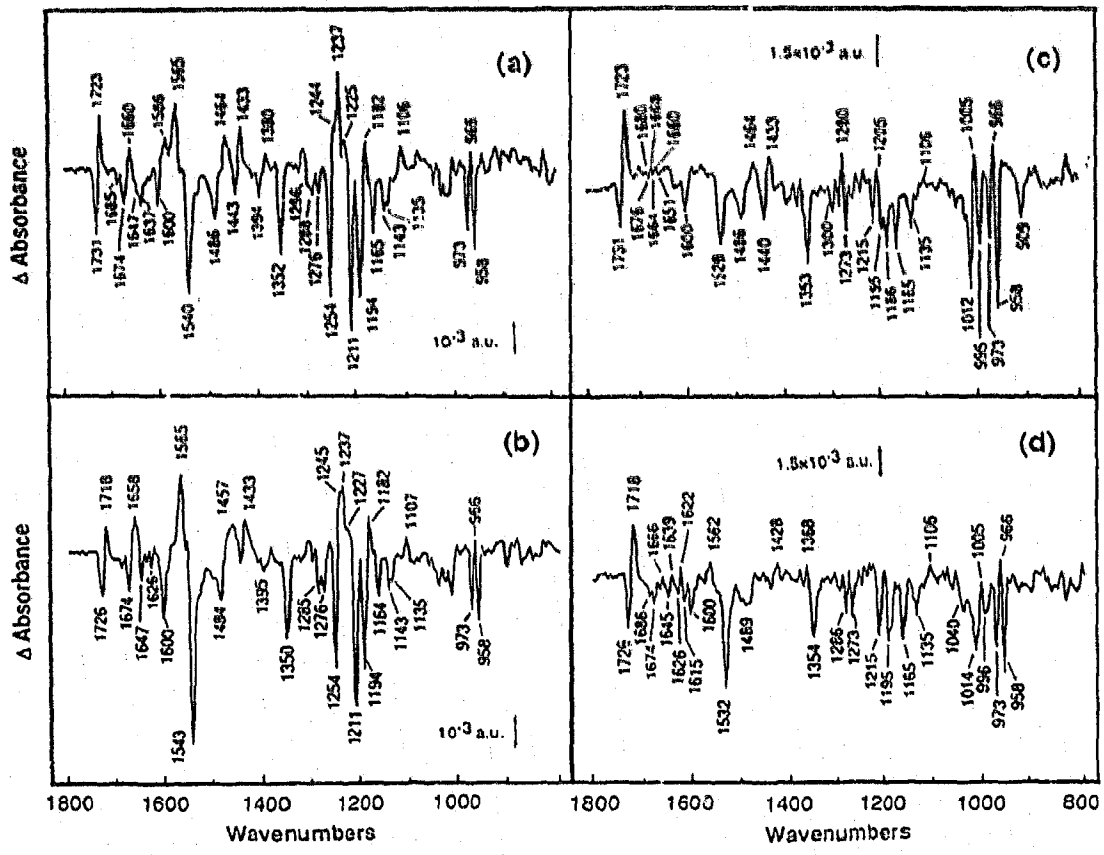

Fig. 1. FTIR difference spectra between Rc and lumiRc. Positive and negative absorption are due to absorption of lumiRc and Rc, respectively. (a) Unmodified sample with H_2O; (b) unmodified sample with D_2O; (c) C_{15}-*d* analog with H_2O; and (d) C_{15}-*d* analog with D_2O.

halogen lamp light source (Unisoku, Japan) for 3 min passed through 550 nm interference filter and 530 nm high pass filter (Toshiba, Japan) from ZnS window side at each temperature. Prior to FTIR measurements, presence of the photoproducts were confirmed by UV-visible absorption spectra with a Shimadzu 2100 spectrophotometer (Shimadzu, Japan).

Difference spectra between retinochrome (Rc) and lumiRc (or metaRc) were obtained as follows: 512 scans of the empty sample holder in the cryostat were collected at 83K (or 270K) as background; 512 scans of Rc at 83K (or 270K) were collected and a spectrum was obtained by calculating the ratio of the sample to the background; scans were repeated until spectra from two successive scans were virtually identical; the average of these last two spectra was employed as Rc spectra; after illumination at 83 K (or 270 K), the same procedure was performed on the photoproducts; Rc/lumiRc (or metaRc) difference spectra in 2 cm^{-1} of resolution were obtained by subtraction of the Rc-spectra from the lumiRc-spectrum (or metaRc-spectrum) recorded at identical temperatures.

3. RESULTS

Difference FTIR spectra between Rc and lumiRc in H_2O and in D_2O are shown in Fig. 1. Difference spectra of the original membrane protein pigment were virtually identical to those of the regenerated pigment with all-*trans*-retinal.

In the region between 1800 and 1700 cm^{-1}, absorption bands for Rc at 1731 cm^{-1} and lumiRc at 1723 cm^{-1} were observed. Both bands shifted about 5 cm^{-1} to 1726 and 1718 cm^{-1}, respectively, after H/D exchange. In the spectra of metaRc (Fig. 2), a broad band at 1708 cm^{-1} in H_2O and 1702 cm^{-1} in D_2O were observed. No other significant absorption band was detectable in this region.

In the region between 1700 and 1600 cm^{-1}, absorption bands at 1685, 1674, 1647 and 1637 for Rc and at 1660 cm^{-1} for lumiRc were observed. The Rc band at 1637 cm^{-1} disappeared by either H/D exchange or C_{15}-*d* substitution and the 1674 cm^{-1} band is insensitive to H/D exchange. The lumiRc band at 1660 cm^{-1} disappeared by C_{15}-*d* substitution and decreased in intensity by H/D exchange leaving a band at 1658 cm^{-1}. In the spectrum of C_{15}-*d* analog in D_2O new bands at 1615 cm^{-1} for Rc and at 1622 cm^{-1} for lumiRc were observed.

Between 1600 and 1500 cm^{-1}, an absorption band for lumiRc at 1565 cm^{-1} is insensitive to H/D exchange and a broad absorption for Rc, centered at 1540 cm^{-1}, shifted to 1543 cm^{-1} in D_2O. In the spectrum of the C_{15}-*d* analog, the Rc band at 1528 cm^{-1} in H_2O shifted to 1532 cm^{-1} in D_2O.

In the region between 1500 cm^{-1} and 1300 cm^{-1}, 4 absorption bands were observed for Rc: 1485, 1443,

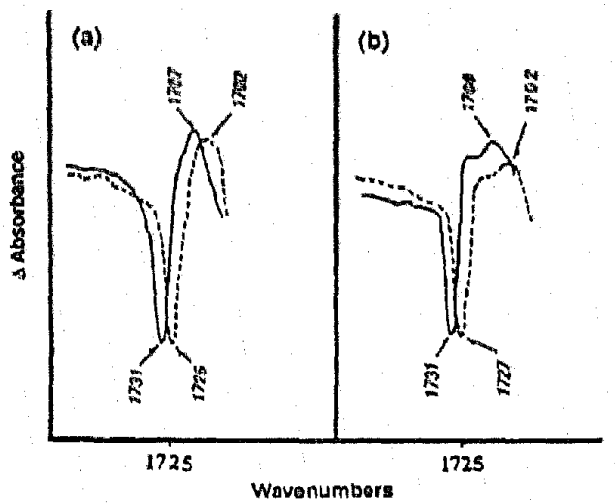

Fig. 2. FTIR difference spectra between Rc and metaRc. Positive absorption is due to absorption of metaRc. (a) Unmodified sample; and (b) C_{15}-*d* analog. Solid line: with H_2O; dotted line: with D_2O. Only the spectrum between 1800 and 1700 cm^{-1} is shown.

1394 and 1352 cm^{-1}. The strong band at 1352 cm^{-1} slightly shifted to 1350 cm^{-1} by H/D exchange, and its absorption maxima of corresponding band in C_{15}-*d* analog in H_2O and in D_2O at 1353 cm^{-1} and 1354 cm^{-1}, respectively. For lumiRh, weak bands at 1464, 1433 and 1380 cm^{-1} were observed. The 1464 cm^{-1} band disappeared by H/D exchange.

In the region 1300 and 1100 cm^{-1}, the following absorption bands for Rc were observed: 1296, 1288, 1276, 1254, 1211, 1194, 1165, 1143 and 1135 cm^{-1}. The bands insensitive to H/D exchange, 1254, 1211, 1194 and 1143 cm^{-1}, disappeared and sharp bands at 1273, 1215, 1195 and 1186 cm^{-1} appeared in the spectrum of the C_{15}-*d* analog. The 1215 and 1195 cm^{-1} bands possibly exist in the spectrum of the unmodified pigment under the strong bands at 1211 and 1194 cm^{-1}, respectively. The 1273 cm^{-1} band of the C_{15}-*d* analog was insensitive to H/D exchange. The 1186 cm^{-1} band disappeared by H/D exchange. The 1164 and 1135 cm^{-1} bands were insensitive to both H/D exchange and C_{15}-*d* substitution. For lumiRc, bands at 1244, 1237, 1225, 1182 and 1106 cm^{-1} were observed. The strong band at 1237 cm^{-1} decreased in intensity by H/D exchange and disappeared by C_{15}-*d* substitution. The band at 1182 cm^{-1} is insensitive to H/D exchange but disappeared by C_{15}-*d* substitution. The weak 1106 cm^{-1} band was seen in all spectra. In the spectrum of the C_{15}-*d* analog, absorption bands at 1280 and 1205 cm^{-1} appeared. The latter band could be detected after the disappearance of the strong bands at 1211 and 1194 cm^{-1} for native Rc by C_{15}-*d* substitution.

In the region between 1100–900 cm^{-1}, Rc has two strong bands at 973 and 958 cm^{-1} which were insensitive to H/D exchange and to C_{15}-*d* substitution. Intensity of a small Rc band at 1015 cm^{-1} increased by H/D exchange. In the spectrum of C_{15}-*d* analog, new bands at 1012 and 995 cm^{-1} were observed. The 1012 cm^{-1} band disappeared by H/D exchange. LumiRc has a band at 966 cm^{-1} and an additional band at 1005 cm^{-1} was observed in the C_{15}-*d* analog.

4. DISCUSSION

The absorption bands in 1800–1700 cm^{-1} of the difference spectra of retinal proteins, which are sensitive to H/D exchange, can be ascribed to the C=O stretching vibrations of carboxylic acids of amino acid residues that are subjected to changes in the proton environment during photoreaction [7,8,9]. Propionic acid shows the C=O stretching at 1780 cm^{-1} in gas phase, at 1760 cm^{-1} in highly diluted non-polar solvent, at 1730 cm^{-1} in dioxane, 1720 cm^{-1} in alcohol and at 1710 cm^{-1} when neat [10]. The C=O stretchings of carboxylic acid in 1780–1760 cm^{-1} (1767, 1772 and 1763 cm^{-1}) for Rh, lumiRh and M intermediate of bR, respectively) are considered to be those which are not hydrogen bonded. The bands appear at lower frequency (1741 cm^{-1} for lumiRh, 1734 cm^{-1} for Rh, 1734, 1743 and 1738 cm^{-1} for bR) are considered to be those which are weakly hydrogen bonded. The shift of bands from 1731 through 1723 to 1708 cm^{-1} for retinochrome series represents that the proton environment of a weakly hydrogen bonded carboxylic acid changes into an environment which causes a stronger hydrogen bond along the reaction, or that one of the carboxylic acids in a different environment gets protonated in each species. Ganter et al. [9] assigned H/D exchange insensitive absorption bands at quite similar wave numbers, 1725 cm^{-1} for Rh and 1731.5 cm^{-1} for the photoproduct, lumiRh, to stretching vibrations of a C=O group of a twisted amide bond bound to the N-terminal end of a proline. The down-shift of the band during the proceeding of the reaction reflects release of the twisting of an amide bond in the protein during the photoreaction. But these bands are insensitive to H/D exchange while the bands for Rc series are sensitive.

The region between 1700 and 1600 cm^{-1}, C=N stretching of the Schiff base linkage of the chromophore is expected. A strong amide I band of the whole protein molecule also appears in this region. Subtle changes in peptide bonds of the protein molecule may yield complex differences in this region [11]. Among the many bands in the region, the Rc band at 1637 cm^{-1} in H_2O and at 1626 cm^{-1} in D_2O could be assigned to C=N stretching of the protonated Schiff base of Rc. The band is similar in frequency to the C=N stretching of the protonated Schiff base in lumiRh [9] and bR [7]. The lumiRc band at 1660 cm^{-1} whose shape was modified by both H/D exchange and C_{15}-*d* substitution, may be assigned to the C=N stretching band of the protonated Schiff base of lumiRc. This band is similar in frequency to the corresponding band in Rh [7,9].

In 1600–1500 cm^{-1}, the C=C stretching bands of the retinal chromophore are expected to be observed, and similar to the region of C=N stretching, absorption bands due to conformational changes of the protein back-bone which are sensitive to H/D exchange appear in this region [12]. Down-shift of Rc 1540 cm^{-1} band to 1528 cm^{-1} by C_{15}-*d* substitution agreed with the observation reported by bathoRh C=C stretching [7]. The lumiRc 1565 cm^{-1} band disappeared by C_{15}-*d* substitution. An explanation for this disappearance is that this band is a C=C stretching mode and shifted to 1540 cm^{-1} where a part of the Rc band remained and cancelled the lumiRc band shifted from 1565 cm^{-1}. Disappearance of the lumiRc 1586 cm^{-1} band both by H/D exchange and C_{15}-*d* substitution suggests that the band is associated with both C_{15}-H and also with the Schiff base or the protein moiety. The slight upshift of the 1540 cm^{-1} band to 1543 cm^{-1} could be caused by H/D exchange of the protein back bone.

An absorption band at 1350 cm^{-1} has been reported for bR. In resonance Raman [13] and FTIR studies [14], the H/D exchange-sensitive band at 1350 cm^{-1} for bR_{568}, which is a chromophore that is all-*trans* retinal, was assigned to N-H in plane bending. The 1352 cm^{-1} band for Rc, which did not disappear by H/D exchange, can not be the N-H in plane bending. Slight shift of this band by C_{15}-*d* substitution or H/D exchange suggests that this band is due to bonds near by the Schiff base. A band at 1255 cm^{-1} was also observed in the resonance Raman and FTIR studies on bR mentioned above. The resonance Raman 1255 cm^{-1} band, which is sensitive to C_{15}-*d* substitution was assigned to C_{15}-H in plane bending, however the complicated behavior of the FTIR 1255 cm^{-1} band by deuteration did not lead to conclusive assignment of the band. The 1254 cm^{-1} band for Rc which disappeared by C_{15}-*d* substitution like the resonance Raman band for bR could be the C_{15}-H in plane bending. Appearance of the 1277 cm^{-1} band by C_{15}-*d* substitution gives another possibility that the 1254 cm^{-1} band shifted to the 1273 cm^{-1} band by C_{15}-*d* substitution, leading to the assignment of the band to a C-C stretching coupled with C_{15}-H vibration.

Absorption band at 1237 cm^{-1} has been observed in the FTIR spectrum of lumiRh [9] and in the resonance Raman spectrum of bathoRh [15]. In FTIR, the 1238 cm^{-1} band was due to C_{12}-C_{13}, C_{14}-C_{15} stretching, the N-H, C_{15}-H and C_{14}-H bending vibrations, because the band is sensitive not only to C_{15}-*d* substitution also by H/D exchange, $^{13}C_{14}$, $^{13}C_{15}$, and C_{14}-*d*-substitution. In the resonance Raman spectrum, H/D exchange insensitive band at 1237 cm^{-1} was assigned to C_{12}-C_{13} stretching vibration of the 11-*cis* chromophore in Rh mainly because it upshifted 82 cm^{-1} on deuteration on C_{14} of the chromophore. The H/D exchange sensitive 1237 cm^{-1} band for lumiRc can not be a C_{12}-C_{13} stretching vibration. Disappearance of the band by C_{15}-*d* substitution and by H/D exchange suggests that the band could be C_{15}-H in plane bending mode. However assignment of vibration frequency without isotopic labelling on each carbon and hydrogen is almost impossible, several absorption bands between 1250–1100 cm^{-1} were assigned to C-C stretching bands of the chromophore by comparison to those frequencies reported for 11-*cis*-Rh and all-*trans*-bathoRh in the resonance Raman studies [15]. For Rc, the 1215, 1164 and 1211 (or 1194) cm^{-1} were assigned to C_8-C_9, C_{10}-C_{11}, and C_{14}-C_{15} stretching. The C_{12}-C_{13} stretching absorption band which is expected to be around 1240 cm^{-1} could not be found. Down-shift of either the band at 1211 or 1194 cm^{-1} to 1186 cm^{-1} by C_{15}-*d* substitution is reasonable for the C_{14}-C_{15} stretching. For lumiRc, the bands at 1205, 1106, 1244, and 1181 cm^{-1} were assigned to C_8-C_9, C_{10}-C_{11}, C_{12}-C_{13}, and C_{14}-C_{15} stretching. However, those absorption frequencies for lumiRc are somewhat different from the reported values for Rh (1217, 1098, 1244 and 1190 cm^{-1}, respectively), the 1181 cm^{-1} band for the C_{14}-C_{15} stretching disappeared by C_{15}-*d* substitution. The C_{10}-C_{11} stretching bands at 1164 cm^{-1} for Rc and at 1106 cm^{-1} for lumiRc reflect 11,12-*trans*- and 11,12-*cis*-geometry of the chromophore in Rc and lumiRc, respectively.

Below 1100 cm^{-1}, absorption bands due to C-H out of plane bending of polyene [10] are expected. Those bands at 973 and 958 cm^{-1} show the presence of two *trans*-double bonds, $C_7=C_8$ and $C_{11}=C_{12}$, in good agreement with the all-*trans*-geometry of the chromophore in Rc. The absorption band at 966 cm^{-1} for lumiRc could be C-H out of plane bending of $C_7=C_8$. The absorption bands at 1012 and 995 cm^{-1} for Rc and at 1005 cm^{-1} for lumiRc in the spectra of C_{15}-*d* analog might be C_{15}-D vibration mode.

Although for FTIR difference spectra in this experiment do not show all absorption bands for each species and assignment of the bands is not conclusive, the shift of the C=O stretching band from Rc through lumiRc to metaRc informs the characteristic change of amino acid side-chain in the protein, the bands detected in the finger-print region seem to reflect an all-*trans* nature of the chromophore in Rc and an 11-*cis* nature in lumiRc, and the existence of H/D exchange sensitive bands for Rc and lumiRc in C=N stretching region could represent that Schiff base in both species is protonated.

Acknowledgement: The authors thank to Professor Teizo Kitagawa of the Institute of Molecular Science for useful suggestions.

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