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Infrared studies of octopus rhodopsin

Existence of a long-lived intermediate and the states of the carboxylic group of Asp-81 in rhodopsin and its photoproducts

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The infrared absorption spectra of octopus rhodopsin and its photoproducts have been observed at 282K and 210K under irradiation of blue and orange light in a neutral condition. The acid metarhodopsin-minus-rhodopsin and lumirhodopsin-minus-rhodopsin difference spectra have been obtained. A new intermediate (called transient acid metarhodopsin) with a lifetime of about 5 s has been found to exist prior to acid metarhodopsin. The present results, together with the data obtained previously, give information on the state of the carboxylic group in the side chain of Asp-81, which is the only acidic amino-acid residue in the part of opsin buried inside the membrane. This carboxylic group is protonated throughout the transformation series, but its state changes on going from transient acid metarhodopsin to acid metarhodopsin. It is probable that these two photoproducts are different from each other only in the opsin moiety.

Octopus rhodopsin; Acid metarhodopsin; Transient acid metarhodopsin; Lumirhodopsin; Photoreaction; Carboxylic group of Asp-81

1. INTRODUCTION

Rhodopsin is a retinal protein which, upon absorption of light, triggers the phototransduction process in both vertebrate and invertebrate photoreceptors. Intermediates in the primary photoreaction and subsequent thermal transformations of rhodopsin have been studied extensively for the visual pigments derived from both vertebrates and invertebrates [1–4]. Among the intermediates, metarhodopsin II of vertebrates and acid metarhodopsin of cephalopods are believed to activate G-proteins, thereby initiating the phototransduction cascade. This physiological function of the intermediates is suppressed by phosphorylation of the serine or threonine residues near the C terminus and subsequent binding of arrestin. The activation of G-proteins by photolyzed rhodopsin is considered to be associated with conformational changes induced in the apoprotein (opsin).

Fourier-transform infrared spectroscopy provides a powerful tool for studying the side-chain structure and main-chain conformation of the apoprotein as well as structural changes in the retinal moiety including the protonation/deprotonation of the Schiff-base linkage [5–8]. Differences between the infrared spectra of bovine rhodopsin and its photoproducts at 70K (bathorhodopsin and isorhodopsin) have been observed and discussed by utilizing the data obtained for samples with selectively isotope-labeled retinals [9]. A similar study on the difference spectra between bovine rhodopsin and its lumirhodopsin has also been performed [10]. Refolding during the dissociation of bovine metarhodopsin II into retinal and opsin has been discussed [11]. The infrared spectra of octopus rhodopsin and its photoproducts at 85K (bathorhodopsin and isorhodopsin) have been observed and compared with the bovine case [12].

The above infrared studies on rhodopsin and its photoproducts have indicated that structural changes in the retinal and opsin moieties occur in the early stages of the transformation series. It is expected that major structural changes in the opsin occurring in later transformation steps would play an important role in initiating the phototransduction cascade by activating the G-proteins. Octopus rhodopsin is an excellent material to study such structural changes upon light irradiation, because the final photoproduct (acid metarhodopsin) is stable at physiological temperatures, and rhodopsin can be almost completely photoregenerated from acid metarhodopsin at neutral pH [13] as well as from any other intermediate. Octopus rhodopsin is known to have at least three intermediates (bathorhodopsin, lumirhodopsin, and mesorhodopsin) between rhodopsin and acid metarhodopsin [4]. In order to gain an insight into the possible structural changes, we have studied differences between the infrared spectra of octopus rhodopsin and acid metarhodopsin. In the course of this

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study, we have found a long-lived precursor of acid metarhodopsin. To specify steps in the transformation series where structural changes occur in the opsin moiety, we have observed the difference spectrum between rhodopsin and an early intermediate (lumirhodopsin) as well.

2. MATERIALS AND METHODS

Octopus (Mizudako, *Paroctopus defleini*) microvillar membranes were prepared in the manner described previously [13]. Deuteration of exchangeable protons in the microvillar membranes was performed as follows. About 300 μl of a D_2O buffer solution (10 mM Tris-DCl, 10 mM $MgCl_2$, pD 6.9) was added to about 1 ml of a suspension ($\approx$90 μM) of the microvillar membranes, and the mixture was centrifuged at 16,000 rpm. The supernatant was exchanged with the D_2O buffer solution, and the resultant suspension was centrifuged. The same procedure was repeated once again.

The equilibrium between rhodopsin and acid metarhodopsin can be reversibly shifted at 282K under irradiation of light of appropriate wavelength regions. Octopus rhodopsin and acid metarhodopsin have their electronic absorption maxima at 476 and 514 nm, respectively [13]. Blue-violet light (wavelength 400–440 nm) for inducing the phototransformation from rhodopsin to acid metarhodopsin and orange-yellow light (wavelength > 540 nm) for the reverse phototransformation were obtained with glass filters, Toshiba V-44 and O-54, respectively. A projector lamp was used as the light source. In the following part of this paper, the colors of the two kinds of lights are simply called blue and orange, respectively.

Since the transformation from lumirhodopsin to mesorhodopsin is suppressed at 210K, lumirhodopsin is in effect the final photoproduct of the forward transformation at this temperature. Lumirhodopsin has its electronic absorption at about 490 nm. Accordingly, the same set of glass filters as employed above could be used for reversibly inducing the phototransformation between rhodopsin and lumirhodopsin.

Infrared spectra were obtained on a JEOL JIR-100 Fourier-transform infrared spectrophotometer equipped with an MCT detector (Judson) at 4 cm^{-1} resolution. The sample (concentrated suspension, pH $\approx$ 7) was placed with a 15 μm thick spacer between two CaF_2 plates, and kept at 282K in a thermostat (Yamato BL-61) for measuring spectral differences between rhodopsin and acid metarhodopsin. The same sample was cooled to 210K in a cryostat (Oxford DN 704) for measuring spectral differences between rhodopsin and lumirhodopsin.

All infrared spectral measurements were carried out under irradiation of either the blue or orange light. First, interferograms from x scans (x = 50, 100, 150, 200, and 250) were accumulated under irradiation of the blue light and stored. The time needed for 50 scans was 25 s. Then, interferograms from the same number of scans were accumulated under irradiation of the orange light. The interferograms accumulated under irradiation of the two different colors were Fourier-transformed separately. This cycle was repeated y times, y depending on x (see figure captions), and the resultant two kinds of spectra were averaged separately. Differences between the two kinds of averaged spectra were then computed. The reason why we employed such a method for obtaining the difference spectra will be explained below.

3. RESULTS

Infrared measurements at 282K were performed under *alternate* irradiation of blue and orange light by changing the number of scans in one acquisition cycle, as described in the preceding section. Differences between the infrared spectra (1,800–1,500 cm^{-1}) observed at 282K under irradiation of blue light and those observed under irradiation of orange light are shown in Fig. 1, where difference spectra a–e were obtained from measurements with 50, 100, 150, 200, and 250 scans in one acquisition cycle, respectively. The difference spectrum changes as the number of scans increases from 50 to 200, but difference spectra d and e obtained from 200 and 250 scans, respectively, are almost identical, indicating that a photostationary state has been attained at 200 scans (equivalent to 100 s). It should be mentioned that a difference spectrum (not shown) obtained by subtracting the spectrum of dark-adapted rhodopsin from

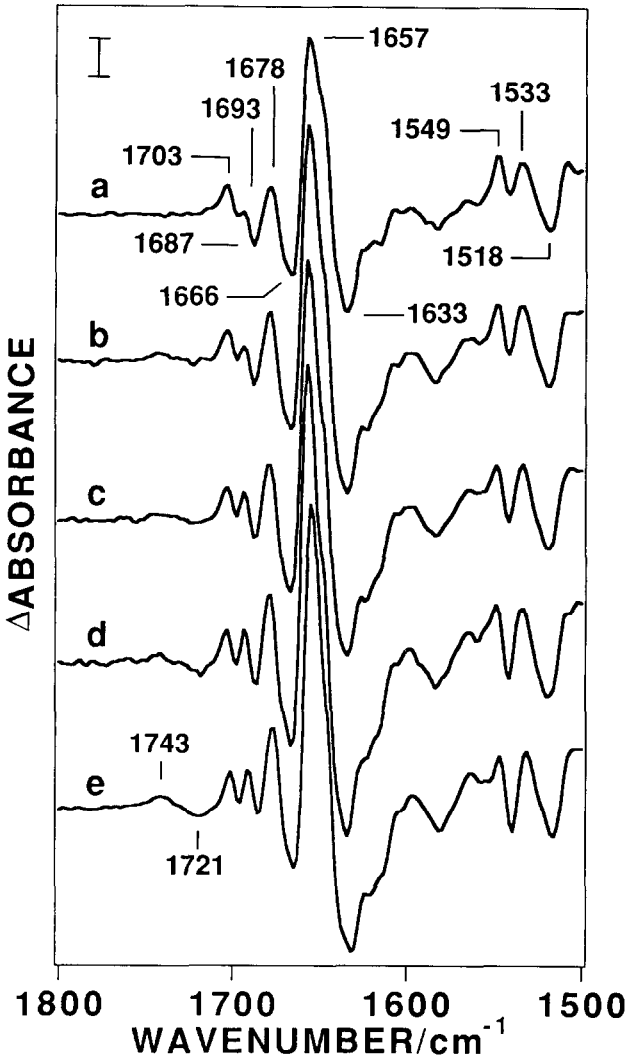

Fig. 1. Differences between the infrared spectra of microvillar membranes containing octopus rhodopsin (suspension in D_2O, pD $\approx$ 7) at 282K observed under irradiation of blue light and those observed under irradiation of orange light. The number of scans (x) in one acquisition cycle: 50, 100, 150, 200 and 250 for difference spectra a–e, respectively. The number of acquisition cycles (y): 300, 57, 40, 22 and 32 for difference spectra a–e, respectively. The absorbance scale indicated by the bar: 1×10^{-4} for a; 2×10^{-4} for b, c, and d; 3×10^{-4} for e. The positive side corresponds to acid metarhodopsin and the new intermediate, and the negative side to rhodopsin.

that observed after 5 min *continuous* irradiation of blue light is essentially the same, except for the spectral quality, as difference spectrum e in Fig. 1. The method of measurements described in the preceding section has ensured the acquisition of high-quality difference spectra with respect to the signal-to-noise ratio and the baseline flatness.

The difference spectrum observed at 210K with 250 scans in one acquisition cycle is shown in Fig. 2.

4. DISCUSSION

4.1. *Existence of a new, long-lived intermediate at 282K*

An infrared difference spectrum obtained by subtracting the spectrum observed at 282K under irradiation of orange light from that observed at the same temperature under irradiation of blue light is expected to correspond to differences between acid metarhodopsin and rhodopsin. Other intermediates in the transformation series, viz., bathorhodopsin, lumirhodopsin, and mesorhodopsin, can be disregarded, because their concentrations must be unmeasurably small due to their short lifetimes at 282K [13].

If the expectation described above is correct, the difference spectra should not depend upon the number of scans (in other words, duration of irradiation), although band intensities may be enhanced with increasing number of scans. The observed clear dependence on the number of scans indicates that an intermediate with a very long lifetime exists either in the forward transformation series leading to acid metarhodopsin or in the reverse process from acid metarhodopsin to rhodopsin. Since this intermediate can be observed only when the acquisition cycle is relatively short (25–75 s equivalent to 50–150 scans), its lifetime is estimated to be about 5 s by a simple kinetic analysis.

It is unlikely that the new intermediate is derived from acid metarhodopsin in the reverse transformation process, since Ogasawara et al. [14] observed three intermediates in the reverse process in a neutral solution of solubilized octopus rhodopsin but their lifetimes were shorter than 1.5 ms. Previously, one of the present authors suggested that, under alkaline conditions, a precursor of octopus alkaline metarhodopsin with a lifetime of several seconds existed [13]. Since this precursor showed a visible absorption spectrum similar to that of acid metarhodopsin, it was called the 'transient acid metarhodopsin'. It is most probable that the new long-lived intermediate under neutral conditions is essentially the same as the 'transient acid metarhodopsin' in an alkaline condition. Accordingly, hereafter the new intermediate will be called transient acid metarhodopsin.

Next, we have tried to determine the difference between the infrared spectra of transient acid metarhodopsin and acid metarhodopsin. For this purpose, a difference between difference spectra a and e in Fig. 1 has been computed (a minus c) by normalizing the intensities of bands in the two difference spectra at the bands in the 1,250–1,200 cm^{-1} region (not shown), which are due to the retinal moiety [9,10,12]. The resultant difference spectrum is shown in Fig. 3, where the positive and negative bands are considered to arise, respectively, from transient acid metarhodopsin and acid metarhodopsin.

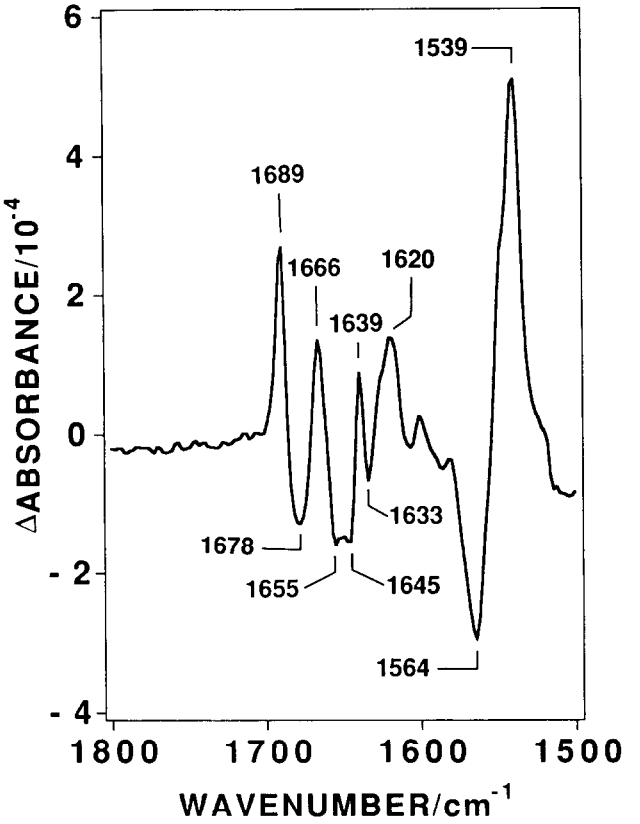

Fig. 2. Difference spectrum obtained at 210K. The sample (suspension in D_2O, pD ≈ 7) and the method of measurement are essentially the same as those employed in obtaining the spectrum in Fig. 1 (*x*, 250; *y*, 72). The positive and negative sides correspond, respectively, to lumirhodopsin and rhodopsin.

4.2. *Bands in the 1,800–1,700 cm^{-1} region and the state of the carboxylic group in the side chain of Asp-81*

A notable feature in the difference spectrum in Fig. 1e is the presence of the weak positive band at 1,743 cm^{-1} and the weak negative band at 1,721 cm^{-1}. Both these bands are assignable to the protonated carboxylic group in the protein side-chain [9,15–17]. These bands slightly shift to lower wavenumbers upon H/D substitution as shown in Fig. 4, where the 1,800–1,700-cm^{-1} region of the difference spectrum in Fig. 1e is expanded and compared with the difference spectrum obtained from microvillar membranes in H_2O suspension. The observed downshifts (about 2 cm^{-1} for both bands) are smaller than the values (about 10 cm^{-1}) reported for the

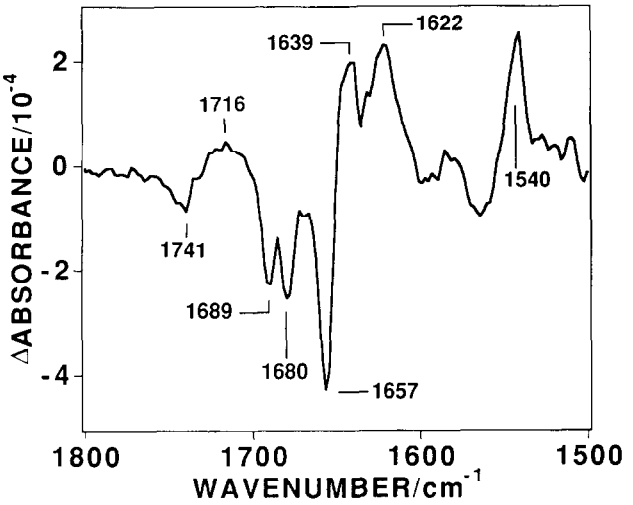

Fig. 3. Difference spectrum obtained by subtracting difference spectrum e from difference spectrum a in Fig. 1. The positive and negative sides correspond, respectively, to transient acid metarhodopsin and acid metarhodopsin.

protonated carboxylic groups in bacteriorhodopsin, bovine rhodopsin, and their photointermediates [9,16,17]. Ganter et al. [16] assigned the 1,725-cm^{-1} band of bovine rhodopsin to the amide-I mode of a distorted peptide group involving a proline residue, on the ground that it showed a shift of only 2 cm^{-1} upon H/D substitution. Very recently, however, Maeda et al. [17] reported that the 1,748-cm^{-1} band due to the carboxylic group of Asp-96 of bacteriorhodopsin L intermediate showed an H/D shift of only 1 cm^{-1}. Therefore, the magnitude of H/D shift cannot be used as an absolute criterion for discerning the origin of a band in the 1,800–1,700 cm^{-1} region.

Octopus rhodopsin has only one aspartic-acid residue (Asp-81) and no glutamic-acid residue in the part of opsin buried inside the microvillar membrane [18]. Accordingly, it is reasonable to consider that the 1,743-and 1,721-cm^{-1} bands arise from the carboxylic group of Asp-81, which is protonated in both rhodopsin and acid metarhodopsin but is located in different environments in these two species.

The fact that the carboxylic group of Asp-81 is protonated means that (i) it does not act as the proton donor for the Schiff base of the retinal moiety in rhodopsin and acid metarhodopsin, both of which are considered to have the protonated Schiff base [19,20], and (ii) there is little possibility of the existence of negatively charged groups in the vicinity of the chromophore, except for anions like Cl^- of extrinsic origin. This makes it difficult to explain the change in the visible absorption on going from octopus rhodopsin to acid metarhodopsin by the external point-charge model [21,22], which is based on the electrostatic interaction between the chromophore and two negative charges, one on the counterion of the protonated Schiff base and the other on a group near the polyene chain of the chromophore. In any case, it is unlikely that Asp-81 is associated with the color regulation of octopus rhodopsin.

The presence of the 1,716-cm^{-1} positive band in the difference spectrum in Fig. 3 indicates that the carboxylic group of Asp-81 of transient acid metarhodopsin is also protonated and is located in an environment similar to that in rhodopsin. On the other hand, no bands are observed in the 1,800–1,700 cm^{-1} region of the lumirhodopsin–rhodopsin difference spectrum in Fig. 2. This applies also to the bathorhodopsin–rhodopsin and isorhodopsin–rhodopsin difference spectra reported by Bagley et al. [12]. Although the mesorhodopsin–rhodopsin difference spectrum has not yet been obtained, it may be concluded from the above results that the carboxylic group of Asp-81 is protonated throughout the transformation series, and the state of this group remains essentially the same until a change occurs in the process of transformation from transient acid metarhodopsin to acid metarhodopsin.

4.3. *Bands in the 1,700–1,500-cm^{-1} region and conformational changes*

Bands in the 1,700–1,600-cm^{-1} region arise from the amide-I modes and the C=N stretch of the protonated Schiff base, and those in the 1,600–1,500 cm^{-1} region from the C=C stretches of the retinal polyene chain and possibly the amide-II modes of the parts in the main chain of opsin which are left undeuterated. In addition, some groups like aromatic rings may give rise to bands in the 1,700–1,500-cm^{-1} region.

Among the four kinds of difference spectra (acid metarhodopsin–rhodopsin in Fig. 1e, lumirhodopsin–rhodopsin in Fig. 2, and bathorhodopsin–rhodopsin and isorhodopsin–rhodopsin obtained by Bagley et al. [12]), great differences exist in the 1,700–1,500-cm^{-1} region. It is noted that even the bands due to rhodopsin appearing on the negative side of each difference spectrum are varied among the four difference spectra. This indicates that the infrared bands of rhodopsin and the four photoproducts strongly overlap with each other, and the overlapping bands cancel each other out to a great extent in the difference spectra. Although the observed bands in the difference spectra which escaped the cancellation reflect structural changes in both the retinal and opsin moieties, further studies are needed to extract useful information. However, it is probable that the spectral differences between rhodopsin and the intermediates in the early transformation stages (bathorhodopsin and lumirhodopsin) contain more information on structural changes involving retinal, whereas those between rhodopsin and the product in the final stage (acid metarhodopsin) may give a clue to the elucidation of conformational changes in the opsin moiety. In this context, the spectral differences between transient acid metarhodopsin and acid metarhodopsin shown in Fig.

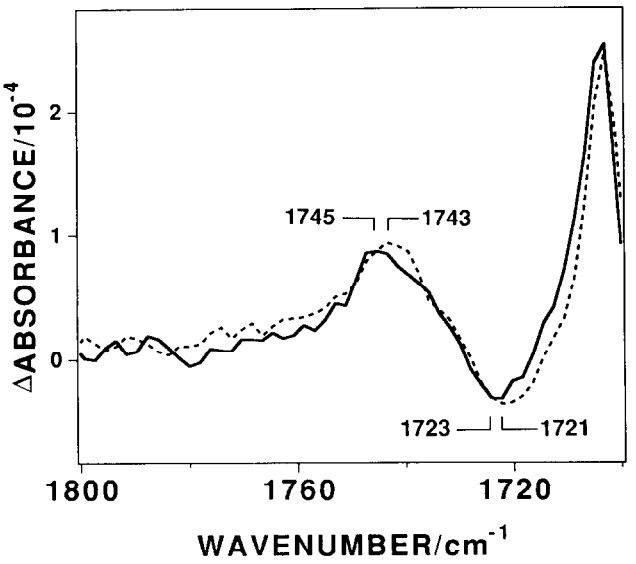

Fig. 4. Expanded difference spectra obtained at 282K. The sample (suspension in either H_2O or D_2O) and the method of measurement are essentially the same as those employed in obtaining the spectra in Fig. 1 (x, 250; y, 24 for H_2O suspension and 32 for D_2O suspension). (——) Suspension in H_2O, pD ≈ 7, (– – –) the same as the difference spectrum in Fig. 1e. The positive and negative sides correspond, respectively, to transient acid metarhodopsin and acid metarhodopsin.

3 are considered to reflect conformational changes occurring in the main chain of the opsin. These conformational changes are probably associated with the change in the state of carboxylic group of Asp-81 discussed above. The previous result that transient acid metarhodopsin was not found in the visible absorption at pH 5.6 [13] suggests that interactions between the retinal and opsin moieties are kept unchanged on going from transient acid metarhodopsin to acid metarhodopsin.

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