Effect of Anion Binding on Iodopsin Studied by Low-Temperature Fourier Transform Infrared Spectroscopy†

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ABSTRACT: The effect of anion binding on iodopsin, the chicken red-sensitive cone visual pigment, was studied by measurements of the Fourier transform infrared spectra of chloride- and nitrate-bound forms of iodopsin at 77 K. In addition to the blue shift of the absorption maximum upon substituting nitrate for chloride, the C═C stretching vibrations of iodopsin and its photoproducts were upshifted 5−6 cm^{-1}. The C═NH and C═ND stretching vibrations were the same in wavenumber between the chloride- and nitrate-bound forms, indicating that the binding of either chloride or nitrate has no effect on the interaction between the protonated Schiff base and the counterion. The vibrational bands of iodopsin in the fingerprint and the hydrogen out-of-plane wagging regions were insensitive to anion substitution, suggesting that local chromophore interactions with the anions are not crucial for the absorption spectral shift. In contrast, bathoiodopsin in the chloride-bound form exhibited an intense $C_{14}H$ wagging mode, whose intensity was considerably weakened upon substitution of nitrate for chloride. These results suggest that binding of chloride changes the environment near the C_{14} position of the chromophore, which could be one of the factors in the thermal reverse reaction of bathoiodopsin to iodopsin in the chloride-bound form.

Iodopsin is one of the four cone visual pigments present in chicken retinas and is known as the most red-shifted natural visual pigment (λ_{max} 571 nm) having an 11-*cis*-retinal chromophore (*1*−*3*). Unlike the other cone visual pigments and rod visual pigment rhodopsin, it has a chloride-binding site in its protein moiety and the binding of chloride causes about a 40-nm red shift of the absorption maximum (*4*−*6*). While the 11-*cis*-retinal chromophore is thought to be embedded in the transmembrane region of the protein, site-directed mutagenesis studies of human red and green, the iodopsin homologues exhibiting a similar chloride effect, demonstrated that amino acid residues responsible for the chloride binding are the histidine (H197) and the lysine (K200), which are present in the second extracellular loop connecting the fourth and fifth transmembrane helices (*7*). Thus it has been a long-standing issue to elucidate the red-shift mechanism due to the chloride binding at such a long distance from the retinal chromophore.

Accumulated evidence has indicated that the chloride-binding site accommodates various monovalent anions other than the chloride, but only the halide ions, including chloride, cause a red shift of the absorption maximum (*8*). Extensive studies using nitrate as a substituting anion demonstrated that nitrate can bind to the same binding site competitively with chloride (*9*). More interestingly, binding of nitrate changes the reaction pathway of bathoiodopsin produced at 77 K (*10*−*12*). That is, like the other cone visual pigments and rhodopsin, it thermally converts to the next intermediate, BL intermediate, and dissociates into *all-trans*-retinal and R-photopsin (protein moiety of iodopsin), whereas it converts back to the original iodopsin via a thermal isomerization of the chromophore when chloride is bound to the binding site. Because absorption characteristics of the original state and the thermal behavior of bathoiodopsin are determined by the electrostatic and steric interaction between the chromophore and a nearby protein, the binding of nitrate could induce the conformation of the protein around the chromophore to be different from that of chloride. Thus we have measured the FTIR[1] spectra of the chloride- and the nitrate-bound forms of iodopsin and their photoproducts at 77 K to identify the chromophore vibrational bands that are different between them. From these results, the molecular mechanism of the spectral red shift and that leading to the unique thermal behavior of bathoiodopsin are discussed.

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from fresh egg yolk; FTIR, Fourier transform infrared; HOOP, hydrogen out-of-plane.

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MATERIALS AND METHODS

Preparation of the Chloride- and Nitrate-Bound Forms of Iodopsin. The chloride-bound form of iodopsin (iodopsin·Cl) was extracted from fresh chicken retinas by a mixture of CHAPS and PC and purified by means of affinity and ion-exchange column chromatography (*13*, *14*). Briefly, the fraction mainly containing iodopsin was eluted from a concanavalin A−Sepharose affinity column (Pharmacia) with buffer A [50 mM HEPES/140 mM NaCl/0.6% CHAPS/phosphatidylcholine (0.8 mg/mL)/20% (w/v) glycerol/0.1 mM phenylmethanesulfonyl fluoride/1 mM dithiothreitol/leupeptin (4 μg/mL)/aprotinin (50 kallikrein inhibitor units/mL)/pH 6.6] supplemented with 100 mM methyl α-D-mannoside. After the NaCl concentration of the fraction was lowered to 10 mM, it was loaded on a carboxymethyl ion-exchange column, from which iodopsin·Cl was eluted with buffer A. To prepare the nitrate-bound form of iodopsin (iodopsin·NO_3), the iodopsin·Cl sample was dialyzed against 100 volumes of the buffer containing 140 mM $NaNO_3$ instead of NaCl with a 5 times exchange of the buffer. It should be noted that the iodopsin·NO_3 can be reversed to the iodopsin·Cl by dialysis against buffer A.

Preparation of 8-D- and 14-D-Iodopsin. 8-D- or 14-D-iodopsin·Cl was reconstituted from R-photopsin (the protein moiety of iodopsin) and 8-D- or 14-D-11-*cis*-retinal, respectively. 8-D- and 14-D-11-*cis*-retinals were prepared as described previously (*15*). R-photopsin in buffer A was prepared as described previously (*16*). After addition of the 1.5−2 times molar excess of each deuterated retinal derivative dissolved in ethanol, the R-photopsin sample was incubated at 4 °C for 1 h.

Preparation of Film Samples for FTIR Spectroscopy. Because iodopsin is unstable in the presence of a high concentration of the detergent CHAPS (*13*), it was incorporated into PC liposome before the film sample was prepared for FTIR spectroscopy. That is, the iodopsin·Cl, iodopsin·NO_3, [8-^{2}H]iodopsin·Cl, or [14-^{2}H]iodopsin·Cl sample was dialyzed against each buffer without CHAPS, PC, and glycerol with a 5 times exchange of the buffer to prepare a liposome. The molar ratio of iodopsin and PC was 1:150−1:250. Liposomes were collected by centrifugation (100000*g* for 1 h) and washed twice with 10 mM NaCl or 10 mM $NaNO_3$ by centrifugation (100000*g* for 1 h). Liposomes containing iodopsins suspended in 10 mM NaCl or 10 mM $NaNO_3$ were then dried on the BaF_2 window. Before the FTIR measurements, the sample was humidified by putting about 0.5 μL of H_2O or D_2O on the BaF_2 window, respectively.

Spectroscopy. FTIR spectra were recorded by a Nicolet 60-SX spectrometer (*17*) or a Bio-Rad FTS-60A/896 spectrometer (*18*). The BaF_2 window on which the sample was dried and humidified was mounted in a cryostat (CF-1204, Oxford) placed in the sample compartment of the FTIR spectrometer. Liquid nitrogen was used as a coolant, and the temperature of the sample was regulated to within 0.1 °C by a temperature controller (ITC-4, Oxford). The sample was irradiated with light from a 1-kW tungsten halogen lamp (Rikagakuseiki). The wavelength of the irradiation light was selected with a glass cutoff filter (Toshiba) or an interference filter (Nihonshinku). For each measurement, 128 interferograms were accumulated. The FTIR spectra shown in the figures are the averages of 8−16 measurements.

UV−vis absorption spectra were recorded with a Shimadzu Model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. The system for the measurements was reported previously (*11*). The percentages of conversion of the bathorhodopsin and the bathoiodopsin·NO_3 by irradiation of the rhodopsin and the iodopsin·NO_3, respectively, at 77 K were estimated in a conventional manner by low-temperature UV−vis absorption spectroscopy (*19*). Briefly, the rhodopsin and iodopsin·NO_3 samples in CHAPS/PC solution containing 66% glycerol and 10 mM hydroxylamine (pH 7.0) were cooled to 77 K and irradiated with 501-nm light until photo-steady-state mixtures were produced. The samples were then warmed to 0 °C and irradiated with >520-nm light until the residual pigments (original pigments and their isopigments, the latter of which were produced by irradiation at 77 K) were completely bleached. The percentages of conversion of bathorhodopsin and bathoiodopsin·NO_3 were estimated to be 52% and 63%, respectively, by comparing the amount of residual pigments with that of the initial pigments present in the samples. Because of the thermal reverse reaction, the percentage of conversion of bathoiodopsin·Cl was difficult to estimate by conventional low-temperature spectroscopy (*10*−*12*). Thus the intensities at 974 and 960 cm^{-1} bands of the iodopsin·Cl were adjusted to those of the bands of the iodopsin·NO_3 (see Figure 4) to obtain the normalized batho/iod·Cl spectrum. This normalization corresponded to the 51% conversion of bathoiodopsin·Cl from iodopsin·Cl upon irradiation at 77 K.

RESULTS

Effect of Substitution of Nitrate for Chloride. The absorption maxima of iodopsin·Cl in CHAPS/PC solution was located at 571 nm, although it was shifted to 530 nm upon substitution of nitrate for chloride. The iodopsin liposomes for the FTIR measurements were then prepared from these samples and were dried on BaF_2 windows and humidified. The difference absorption maxima calculated by subtracting the spectra recorded before and after irradiation of the liposome samples with an orange light (>560 nm) were the same as those in CHAPS/PC solution, although the absolute spectra displayed increased absorbances at shorter wavelengths due to the scattering. Prior to FTIR measurements, the film samples were subjected to low-temperature UV−vis spectroscopy, and it was confirmed that they exhibited photochemical and subsequent thermal reactions identical with those in the CHAPS/PC solution (*11*) (data not shown).

Difference FTIR Spectra of Iodopsin·Cl and Iodopsin·NO_3. The difference FTIR spectra between iodopsin·Cl and their photoproducts at 77 K were recorded as follows: The film sample of iodopsin·Cl mounted in the cryostat was cooled to 77 K and irradiated with 501-nm light for 5 min to produce a photo-steady-state mixture mainly containing bathoiodopsin·Cl. The sample was then successively irradiated with 710-nm light for 5 min, with >610-nm light for 5 min, and with 501-nm light for 5 min. These irradiations caused the formations of photo-steady-state mixtures mainly containing iodopsin·Cl, isoiodopsin·Cl (9-cis form), and bathoiodopsin·Cl (all-trans form), respectively. Before each irradiation, the FTIR spectrum was recorded (average of 128 interferograms), and the difference FTIR spectra between bathoiodopsin·Cl and iodopsin·Cl (batho/iod·Cl), isoiodopsin·Cl and iodopsin·Cl (iso/iod·Cl), and bathoiodopsin·Cl and isoiodopsin·

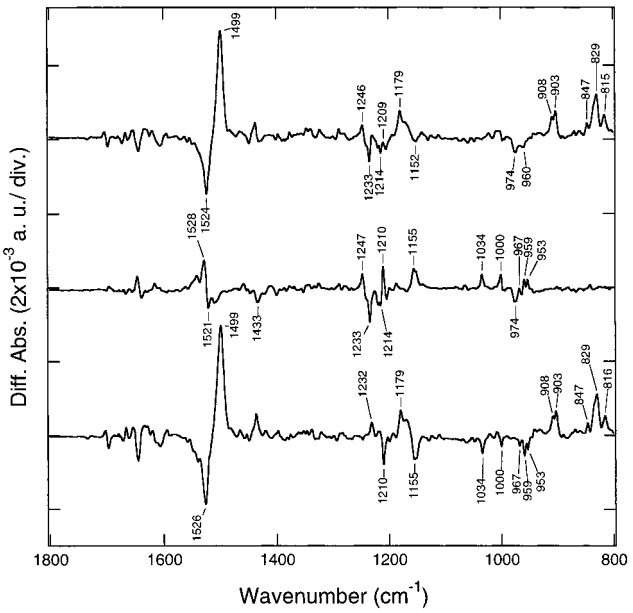

FIGURE 1: Difference FTIR spectra of iodopsin·Cl system in H_2O recorded at 77 K. Difference FTIR spectra between iodopsin·Cl (negative) and bathoiodopsin·Cl (positive) [batho/iod·Cl, upper curve], that between iodopsin·Cl (negative) and isoiodopsin·Cl (positive) [iso/iod·Cl, middle curve], and that between isoiodopsin (negative) and bathoiodopsin (positive) [batho/iso·Cl, lower curve] are shown. The procedures for the measurements of the difference spectra are described in the text.

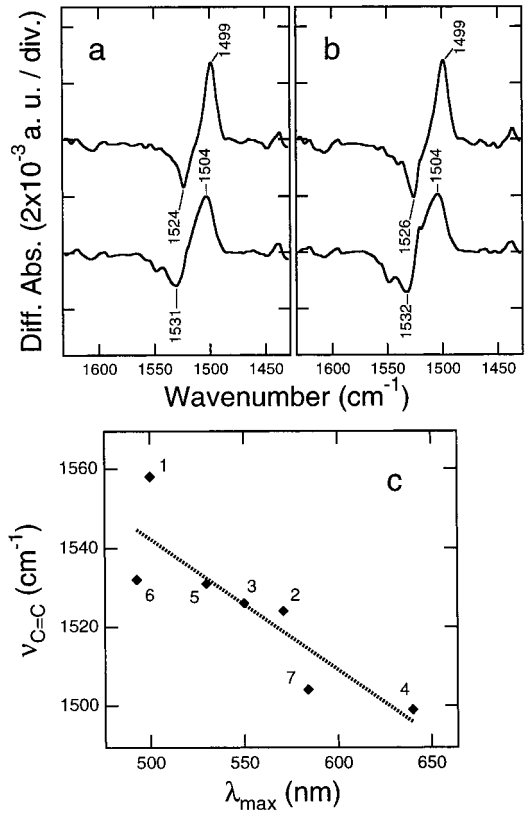

FIGURE 2: Spectral region of C=C stretching vibrations of the difference FTIR spectra. (a) Batho/iod·Cl (upper) and batho/iod·NO_3 (lower) spectra. (b) Batho/iso·Cl (upper) and batho/iso·NO_3 (lower) spectra. (c) Correlation of the ethylenic frequencies of iodopsin·Cl, iodopsin·NO_3, and their photoproducts at 77 K with their absorption maxima: (1) rhodopsin, (2) iodopsin·Cl, (3) isoiodopsin·Cl, (4) bathoiodopsin·Cl, (5) iodopsin·NO_3, (6) isoiodopsin·NO_3, and (7) bathoiodopsin·NO_3.

Cl (batho/iso·Cl) were obtained by calculating the difference spectrum before and after the respective irradiations. The series of irradiation was repeated 10 times, and the data were averaged. The obtained difference FTIR spectra in 1800−800 cm^{-1} region are shown in Figure 1. Similar experiments were carried out with iodopsin·NO_3 film. Because the absorption spectra of iodopsin·NO_3 and its photoproducts are blue-shifted from those of iodopsin·Cl and its photoproducts, the wavelengths of the irradiation light were selected to be 501, >670, and >590 nm for formation of the photo-steady-state mixtures mainly containing bathoiodopsin·NO_3, iodopsin·NO_3, and isoiodopsin·NO_3, respectively. The irradiations were repeated 10 times and the obtained spectra were averaged (Figures 2−4).

C=C Stretching Modes. The bands due to the C=C stretching modes of chromophores in the difference FTIR spectra of iodopsin·Cl and iodopsin·NO_3 in H_2O are shown in Figure 2a,b. The frequencies of iodopsin·Cl, isoiodopsin·Cl, and bathoiodopsin·Cl were located at 1524, 1526, and 1499 cm^{-1}, respectively (upper curves in Figures 2a and b), while those of iodopsin·NO_3, isoiodopsin·NO_3, and bathoiodopsin·NO_3 were at 1531, 1532, and 1504 cm^{-1}, respectively (lower curves in Figure 2a,b). It is widely accepted that the C=C stretching (ethylenic) frequency of the chromophore is inversely correlated with the absorption maximum of the pigment (*20*−*22*). In fact, the observed frequencies are correlated well with the absorption maxima of the pigments estimated by low-temperature UV−vis spectroscopy (*9*−*12*) (Figure 2c).

C=NH and C=ND Stretching Modes. Because one of the mechanisms to account for the red-shifted absorption maximum in visual pigment is the strength of hydrogen bonding of the protonated Schiff base (*23*), the comparison of the C=NH and C=ND stretching frequencies of iodopsin·Cl and iodopdin·NO_3 is interesting (*24*−*26*). Thus we have compared these frequencies of the spectra in H_2O and D_2O in the region from 1730 to 1580 cm^{-1} (Figure 3). Unlike the resonance Raman spectra, the FTIR spectra contain bands originated from peptide carbonyls (amide I) and side-chains of amino acids in addition to the C=NH and C=ND stretching bands in this region. Thus the FTIR difference spectra between respective pigments showed the bands that reflect the conformational changes of the transmembrane helices and/or the side chains induced by the isomerization of the chromophore. However, these bands do not change significantly when deuterated (*27*); the subtraction of the FTIR difference spectrum from that measured after deuteration could remove the bands and thus extract the C=NH and C=ND stretching bands. The subtraction spectrum (lower curve in Figure 3a) obtained from iso/iod·Cl spectra (upper curves in Figure 3a) exhibits two positive and two negative bands due to the C=NH and C=ND bands of the chromophore. The negative 1640 cm^{-1} band and the positive 1618 cm^{-1} band should be assigned to the C=NH and C=ND stretching modes of iodopsin·Cl, because the values are in good agreement with those observed by resonance Raman spectroscopy (1644 and 1621 cm^{-1}, respectively; *28*). Thus the positive 1647 cm^{-1} band and the negative 1629 cm^{-1} band should be the C=NH and C=ND stretching modes of isoiodopsin·Cl. From the subtraction spectrum (lower curve in Figure 3b) obtained from iso/batho·Cl spectra (upper curves in Figure 3b), the C=NH and C=ND stretching

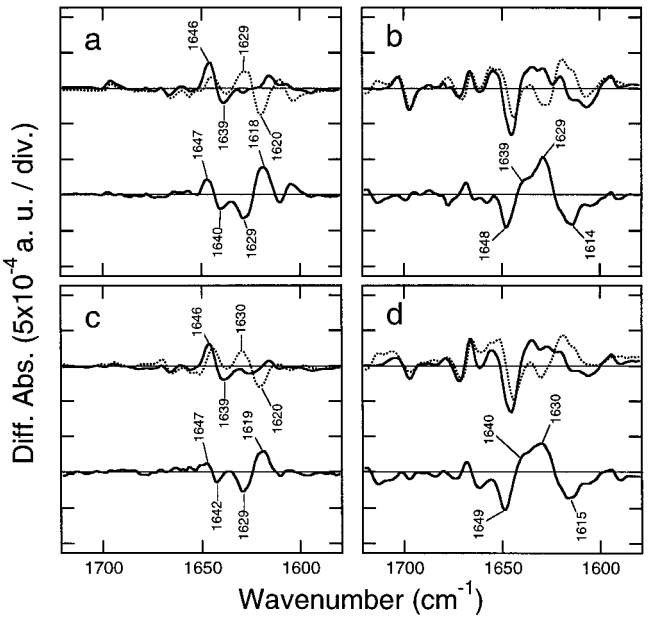

FIGURE 3: Identification of the C═NH and C═ND stretching modes of iodopsin·Cl, iodopsin·NO_3, and their photoproducts at 77 K. (a) Upper curves: iso/iod·Cl spectra in H_2O (solid) and D_2O (dotted). Lower curve: Difference spectrum between iso/iod·Cl spectra in H_2O and D_2O. (b) Upper curves: batho/iso·Cl spectra in H_2O (solid) and D_2O (dotted). Lower curve: Difference spectrum between batho/iso·Cl spectra in H_2O and D_2O. (c) Upper curves: iso/iod·NO_3 spectra in H_2O (solid) and D_2O (dotted). Lower curve: Difference spectrum between iso/iod·NO_3 spectra in H_2O and D_2O. (d) Upper curves: batho/iso·NO_3 spectra in H_2O (solid) and D_2O (dotted). Lower curve: Difference spectrum between batho/iso·NO_3 spectra in H_2O and D_2O.

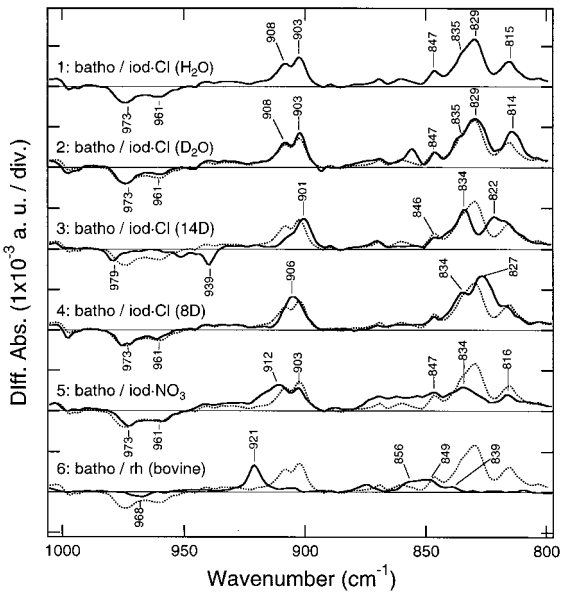

FIGURE 4: Difference FTIR spectra in the HOOP region: (1) batho/iod·Cl spectrum in H_2O; (2) batho/iod·Cl spectrum in D_2O; (3) batho/iod·Cl spectrum recorded with 14-D-iodopsin·Cl; (4) batho/iod·Cl spectrum recorded with 8-D-iodopsin·Cl; (5) batho/iod·NO_3 spectrum; (6) difference FTIR spectrum between bathorhodopsin (positive) and rhodopsin (negative) from bovine retina at 77 K. For comparison of the spectra, the batho/iod·Cl spectrum is shown by the dotted lines in the figure.

frequencies of bathoiodopsin·Cl are assigned to 1639 and 1614 cm^{-1}, which are also in good agreement with those (1640 and 1613 cm^{-1}) obtained from resonance Raman experiments (*28*).

The C═NH and C═ND frequencies of iodopsin·NO_3 and its photoproducts are also identified by similar subtraction procedures (lower curves in Figure 3c,d). The interesting facts are that the subtraction spectra from hydrated and deuterated iso/iod·NO_3 and iso/batho·NO_3 spectra are almost the same in shape as the corresponding spectra obtained from the experiments on the chloride-bound form. These results clearly show that the frequencies are almost identical with those estimated in the chloride-bound form. Thus we concluded that the binding of either chloride or nitrate has no effect on the hydrogen-bonding character of the protonated Schiff base.

C−C Stretching Modes. The C−C stretching modes of the chromophore should appear in the fingerprint region of the FTIR spectra, although several bands that were not identified as the specific modes of the chromophore and/or the protein are superimposed (see Figure 1). The interesting observation is that the difference FTIR spectra derived from iodopsin·NO_3 were almost the same in shape as those from iodopsin·Cl (data not shown), suggesting that irrespective of the assignments, the anion substitution has little effect on the C−C stretching modes of the chromophore in the iodopsin system.

Hydrogen-Out-of-Plane Wagging Modes. The hydrogen out-of-plane (HOOP) modes due to the hydrogens attached to the carbon atoms of the chromophore are expected to be observed in the 1000−800 cm^{-1} region. In contrast to iodopsin·Cl and isoiodopsin·Cl, bathoiodopsin·Cl exhibits several bands in this region (curve 1 in Figure 4), suggesting that the chromophore of bathoiodopsin·Cl is in a twisted conformation, as is that of bathorhodopsin (*28*−*30*). Interestingly, bathoiodopsin·Cl has two bands at 908 and 903 cm^{-1} in the region where only the isolated $C_{11}H$ wag at 921 cm^{-1} was detected in bathorhodopsin (curve 6 in Figure 4). Similarly, the original iodopsin·Cl displays two bands at 973 and 961 cm^{-1}, while rhodopsin displays only one band at 968 cm^{-1} due to the HC_{11}═$C_{12}H$ A_2 HOOP combination (*29*, *30*). These results suggest that some HOOP wagging mode(s) other than the $C_{11}H$ and $C_{12}H$ mode also contributes to the infrared spectra of iodopsin and bathoiodopsin. Previous studies on the normal-mode analysis of the chromophore of bathorhodopsin (*29*) predicted a mixed mode of NH, $C_{15}H$, and $C_{14}H$ waggings to be located at 928 cm^{-1}, which is near the isolated 921 cm^{-1} $C_{11}H$ wagging mode. Thus we first measured the batho/iod·Cl spectrum in the deuterated sample (curve 2 in Figure 4) and found no significant difference in the HOOP region of the spectrum between hydrated and deuterated samples. However, we found that the 908 cm^{-1} band of bathoiodopsin was greatly reduced in the spectrum from iodopsin having 14-D-retinal (curve 3 in Figure 4), indicating that the band is mainly due to the $C_{14}H$ wagging mode. The 973 and 961 cm^{-1} bands of iodopsin also shifted to 979 and 939 cm^{-1}. On the other hand, the spectrum obtained from the iodopsin having 8-D-retinal (curve 4 in Figure 4) showed a similar shape in the region from 1000 to 870 cm^{-1}, although the 908 cm^{-1} band of bathoiodopsin slightly shifted to the lower wavenumber to converge the two bands. These results strongly suggest that the chromophore in the iodopsin system interacts with a protein at the position near C_{14} which is different from that in the rhodopsin system.

In the batho/iod·NO_3 spectrum (curve 5 in Figure 4), the 908 cm^{-1} band of bathoiodopsin·Cl upshifted to 912 cm^{-1}, while the 903 cm^{-1} band was unchanged. The 973 and 961 cm^{-1} bands of iodopsin were almost insensitive to the anion substitution. Thus the anion binding effect might be seen near the C_{14} position of the chromophore in bathoiodopsin. The most remarkable change in the batho/iod spectrum due to the exchange of the anion was seen in the region from 850 to 800 cm^{-1}, where several mixed wagging modes appeared. At least three bands (847, 829, and 816 cm^{-1}) and one shoulder (about 835 cm^{-1}) were identified in the batho/iod·Cl spectrum (curve 1 in Figure 4). Among the bands, the intensity of the band at 829 cm^{-1} was greatly reduced in the batho/iod·NO_3 spectrum (curve 5 in Figure 4). Comparison of the bathoiodopsin spectrum with the FTIR (*27*) and Raman (*29*) spectra of bathorhodopsin suggested that the 829 cm^{-1} band of bathoiodopsin·Cl corresponded to the 849 cm^{-1} band of bathorhodopsin, which was assigned to the $C_{14}H$ and $C_{12}H$ wagging modes (*29*). In fact, the replacement of retinal chromophore by 14-D-retinal in iodopsin·Cl caused a shift of the band to 822 cm^{-1} with decreased intensity (curve 3 in Figure 4), suggesting that the $C_{14}H$ wagging mode would contribute to this band. On the other hand, the replacement by 8-D-retinal caused a slight (2 cm^{-1}) shift of this band with increased intensity. Thus we concluded that the change in the FTIR spectrum of bathoiodopsin upon anion exchange would originate from the environmental change near the C_{14} position of the chromophore.

It should be noted that the intensity of the 903 cm^{-1} band of bathoiodopsin·Cl is comparable to that of the 921 cm^{-1} band of bathorhodopsin (curve 6 in Figure 4), when the percentages of conversion of the original pigments to their bathointermediates are normalized. That is, the 829 cm^{-1} band of bathoiodopsin·Cl is extraordinarily high, so that the intensities of the bands of bathoiodopsin·Cl in the region from 850 to 800 cm^{-1} appear to be higher than those of bathorhodopsin and bathoiodopsin·NO_3. These results suggest that the binding of chloride causes the specific interaction of the C_{14} position of the chromophore with surrounding protein (see Discussion section).

Isoiodopsin·Cl exhibited the specific bands at 1034, 1000, 967, 959, and 953 cm^{-1} in the region below 1050 cm^{-1} (Figure 1). These bands did not change upon substitution of nitrate for chloride. The interesting observation was that the 1034 cm^{-1} band disappeared when 14-D-retinal was incorporated (data not shown). Therefore, like iodopsin, isoiodopsin displays no anion effect, at least near the C_{14} position of the chromophore.

DISCUSSION

Opsin Shift of Iodopsin and Chloride Effect. One of the most remarkable differences between iodopsin and rhodopsin is their absorption maxima. The opsin shift of iodopsin·Cl (λ_{max} 571 nm) is 5200 cm^{-1}, which is ~2500 cm^{-1} larger than that of rhodopsin (λ_{max} 500 nm). We proposed in the previous studies (*16*) that the interaction between the protonated Schiff base of the chromophore and its counterion in iodopsin is weaker than that in rhodopsin. The present study and that reported previously (*28*) clearly showed that the C=NH stretching frequency of iodopsin (1640 cm^{-1}) is about 16 cm^{-1} lower than that of rhodopsin (1656 cm^{-1}) and that the shift of the frequency upon deuteration is about 10 cm^{-1} smaller in iodopsin than in rhodopsin. These results indicated that the weak hydrogen bonding of the Schiff base group in iodopsin is one of the mechanisms for the red-shifted absorption maximum. However, the present study clearly showed that the substitution of the anion has no effect on the hydrogen-bonding character of iodopsin, although it causes a 40-nm shift of the absorption maximum. Thus the difference in hydrogen-bonding character explains only a part of the red shift of the absorption spectrum between rhodopsin and iodopsin.

The ethylenic frequencies that correlate with the absorption maxima are different between iodopsin·Cl and iodopsin·NO_3, but the frequencies of the C−C stretching modes in the fingerprint region and those of the HOOP wagging modes are almost the same in these pigments. Therefore, the shift of the absorption maximum upon substitution of nitrate for chloride is not caused by local protein perturbations along the polyene chain of the chromophore. Because chloride should bind to the histidine or the lysine through electrostatic interaction in the binding site, the chloride bound to the protein would act as a dipole which could perturb the global electronic structure of the chromophore. In this sense, the effect of chloride on the absorption characteristics of the chromophore may be similar to a dipolar effect of the polar amino acid residues which are responsible for the shift of the absorption maximum but exhibit little effect on the chromophore structure (*23*). Because nitrate can bind to the same binding site as chloride (*9*), it may also affect the absorption maximum of iodopsin. The difference in the effect of the absorption maximum between chloride and nitrate may account for the different character of these anions by which a different conformation of the protein moiety would be induced, thereby resulting in a different location of these anions relative to the retinal chromophore (see below).

Chromophore Structure of Bathoiodopsin and Its Thermal Behavior. From the comparison of the spectra in the HOOP region, the difference in chromophore structure between rhodopsin and iodopsin systems was elucidated. Rhodopsin exhibited only one band at 968 cm^{-1} due to the $HC_{11}=C_{12}H$ A_2 HOOP combination (*29*, *30*), while iodopsin exhibited two bands at 973 and 961 cm^{-1}, which shifted to 979 and 939 cm^{-1} upon incorporation of 14-D-retinal. In the region where only the isolated $C_{11}H$ band was observed in bathorhodopsin, the $C_{14}H$ band was also observed in bathoiodopsin. These results suggest the presence of the specific interaction near the C_{14} position of the chromophore in the iodopsin system. There are two possible interpretations for the manner of interaction between the chromophore and a nearby protein. One is that the chromophore is in a twisted conformation around the C_{14} position through a steric interaction with nearby amino acid(s), and the other is an electrostatic (or hydrogen-bonding) interaction between the hydrogen attached to the C_{14} of the chromophore and surrounding amino acid(s).

When chloride was substituted with nitrate, the HOOP modes of iodopsin scarcely changed, but the modes of bathoiodopsin dramatically changed. These results indicated that the binding of anion affects the chromophore conformation of bathoiodopsin. It should be noted that the 829 cm^{-1} band of bathoiodopsin·Cl displayed an extraordinarily high intensity compared to those of the other HOOP bands in

bathoiodopsin·Cl, bathoiodopsin·NO_3, and bathorhodopsin. Because the C_{14}H wagging mode contributes to this band, it is reasonable to speculate that the binding of chloride induces a notable interaction of the C_{14} position of the chromophore with nearby amino acid(s). If one can assume that the high intensity of the 829 cm^{-1} band originates from the twisted conformation around the C_{14} position of the chromophore, this interaction may be one of the motive forces of the reverse reaction from bathoiodopsin·Cl to iodopsin·Cl through rotation of the C_{11}═C_{12} double bond (*12*).

Anion Binding Effect on Iodopsin. From the comparison of the chromophore bands upon anion substitution of the iodopsin sample, we could also speculate about the effect of anion binding on the protein structure around the chromophore. Although we did not observe anion-dependent local perturbations along the polyene chain of the chromophore in the original pigment, local perturbations around the C_{14} position of the chromophore in the batho intermediate were observed. The appearance of the 829 cm^{-1} band with large intensity in bathoiodopsin·Cl suggests that the binding of chloride causes a protein conformational change by which the chromophore tends to be twisted around the C_{14} position. It is reasonable to speculate that, like rhodopsin, iodopsin converts to bathoiodopsin by movement of the half of the polyene chain containing the Schiff base (*31*). Thus, binding of chloride may form a tight chromophore binding site in which the all-trans chromophore of bathoiodopsin is in a highly twisted conformation (*10*), thereby resulting in the reverse reaction to iodopsin. Although it is difficult to explain why the nitrate binding does not induce the conformational change as the chloride binding does, the difference should be explained by the different ionic character of chloride and nitrate. Nitrate is one of the lyotropic anions whose ability for hydration is relatively low, while chloride tends to be hydrated. Therefore, it is an intriguing argument that chloride can bind to the binding site with water molecule(s), so that the binding site tends to be close to the chromophore binding site through the hydrogen-bonding network including water molecule(s), while binding of only a lyotropic nitrate does not induce the conformational change. In fact, our FTIR measurements in the high-frequency region indicated that four water O−H stretching vibrations are changed upon cis−trans isomerization of the iodopsin·Cl, while only two of them were observed in iodopsin·NO_3 (Hirano et al., manuscript in preparation). However, it is evident that the comparative study of anion-free iodopsin with those of the chloride- and nitrate-bound forms is necessary for furthering our understanding of the anion binding mechanism in iodopsin. Thus our future research will investigate the FTIR spectra of the anion-free iodopsin in addition to the complete assignments of the chromophore bands.

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