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Chloride Effect on Iodopsin Studied by Low-Temperature Visible and Infrared Spectroscopies[†]

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ABSTRACT: To investigate the chloride effect on the spectral properties of iodopsin, we have prepared an anion-free iodopsin (iodopsin•free) by extensive dialysis of an iodopsin sample against a buffer containing no chloride, and visible and infrared difference spectra between iodopsin•free and its photoproduct at 77 K were recorded. The absorption maximum of iodopsin•free in L-α-phosphatidylcholine liposomes was 528 nm, which was almost identical with that of the nitrate-bound form of iodopsin (526 nm, iodopsin•NO_3), but 43 nm blue-shifted from that of the chloride-bound form of iodopsin (iodopsin•Cl). The iod/batho visible difference spectrum obtained from iodopsin•free was similar in shape to that from iodopsin•NO_3, but not to that from iodopsin•Cl. FTIR spectroscopy revealed that the chromophore vibrational bands and the peptide bonds of the original state in iodopsin•free were identical with those in iodopsin•NO_3 and were also similar to those in iodopsin•Cl except for the ethylenic vibrations of the chromophore. In contrast, those of the batho state in iodopsin•free were similar to those in iodopsin•NO_3 but considerably different from those in iodopsin•Cl. These results suggested that the binding of chloride but not nitrate induces a conformational change in the protein and that the chloride binding site is situated in a position where it directly interacts with the chromophore when the chromophore is photoisomerized. FTIR spectroscopy also revealed that one of the four water bands observed in the batho/iod spectrum of iodospin•Cl is absent in the spectra of iodopsin•free and iodopsin•NO_3. Thus, in contrast to nitrate, a lyotropic anion, chloride would bind to the binding site with water molecule(s) which could form a hydrogen-bonding network with amino acid residue(s) near the chromophore, thereby resulting in the red shift of the absorption maximum of iodopsin.

Iodopsin (λ_{max} = 571 nm) is a visual pigment present in chicken red-sensitive cones (*1*) and belongs to a family of long-wavelength-sensitive visual pigments among the four families (short, middle1, middle2, and long) of cone visual pigments (*2*, *3*). In contrast to the cone visual pigments of other families and the rod visual pigment rhodopsin, it has a chloride binding site in its protein moiety, and binding of chloride causes a red shift in the absorption maximum (*4*, *5*). The chloride binding site also accommodates various monovalent anions, but most anions except for halide anions induce little shift (*6*) or a blue shift (*7*) of the absorption maximum. Recent extensive studies using nitrate as a substituting anion showed that nitrate can bind to the same binding site competitively with chloride (*8*) but exhibits an ∼40 nm blue shift in the absorption maximum from that of the chloride-bound form. Thus, it is of interest to investigate how the absorption spectrum of iodopsin is regulated by the anion.

In the previous studies (*9*), we have investigated the anion effect on the tuning mechanism of the absorption maximum by comparing the low-temperature FTIR[1] spectra of chloride- and nitrate-bound forms of iodopsin (iodopsin•Cl and iodopsin•NO_3, respectively). The results showed no differences in the C−C stretching and the hydrogen out-of-plane (HOOP) wagging, and the C=NH (and C=ND) stretching bands were observed in the 11-cis form (original state) of these pigments. The difference was observed only in the C=C stretching frequencies, and it correlated with the difference in absorption maxima between these pigments. Thus, the effects of chloride and nitrate originate from a long-range interaction with the chromophore but not from local protein perturbations along the polyene chain of the chromophore. These observations are consistent with the fact that the amino acid residues (H194 and K197) responsible for the chloride binding are situated in the second extracellular loop connecting the fourth and fifth transmembrane helices (*10*) (Figure 1). However, we found that the HOOP wagging bands, especially that containing the $C_{14}H$ wagging mode, of bathoiodopsin, the chromophore photoisomerized state, in the chloride-bound form are considerably different from those in the nitrate-bound form (*9*). Therefore, the binding

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[1] Abbreviations: iod, iodopsin; batho, bathoiodopsin; iso, isoiodopsin; FTIR, Fourier transform infrared; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; Con A, concanavalin A; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; KIU, kallikrein inhibitor units.

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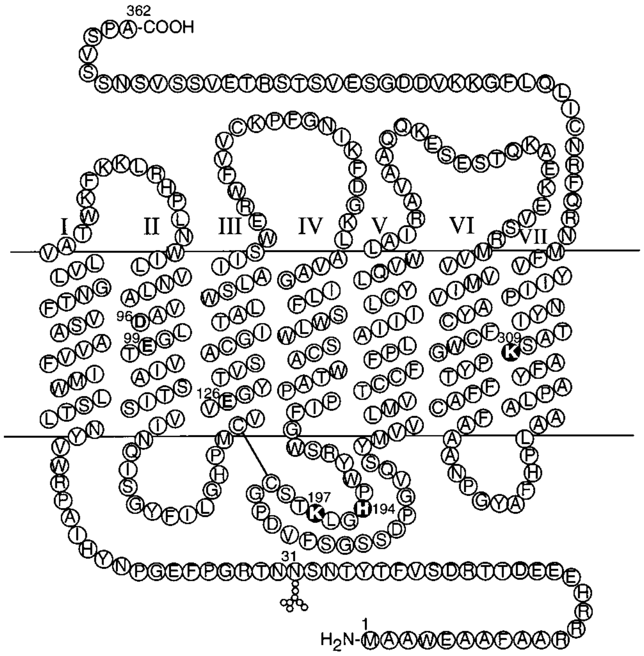

FIGURE 1: Two-dimensional model of iodopsin. The following residues are highlighted: Lys309 forming a Schiff base with 11-*cis*-retinal and His194 and Lys197 as the chloride binding sites of visual pigments which belong to group L (*10*). Membrane-embedded aspartate and glutamate residues are shown in bold letters containing Glu126 that corresponds to Glu113 of bovine rhodopsin.

of the anion could induce the protein conformational change that perturbs the chromophore binding site near the C_{14} position of the chromophore of bathoiodopsin.

The above conclusion was derived from comparative studies with iodopsin•Cl and iodopsin•NO_3. Thus, we were unable to determine which of the conformational changes were derived from the binding of chloride or nitrate. Also, we were unable to detect conformational changes, if binding of the chloride and nitrate was induced to similar extents. Therefore, it is necessary to investigate the effect of each anion by comparing the data for each anion with those obtained from the anion-free iodopsin (iodopsin•free), to further our understanding of the role of chloride. For this purpose, we have prepared an iodopsin•free sample by extensive dialysis of iodopsin•Cl and measured the difference visible and infrared spectra between iodopsin•free and its photoproducts at 77 K. The results showed that the spectra from iodopsin•free were surprisingly similar to those from the iodopsin•NO_3 but considerably different from those of the iodopsin•Cl. Detailed analyses of the FTIR spectra indicated that the binding of chloride but not nitrate induces a conformational change in the protein to situate the binding site near the chromophore binding site, and the binding of chloride perturbs the chromophore structure when it is photoisomerized. Together with the experimental data obtained from the high-frequency region of the FTIR spectra, we will discuss the unique character of iodopsin in the chloride-bound form.

MATERIALS AND METHODS

Sample Preparation. Iodopsin was extracted from about 2000 chicken retinas by a mixture of CHAPS and PC and purified by column chromatography (*11*). In addition to the standard procedure, the following steps were employed to prepare a film sample for FTIR spectroscopy. The iodopsin sample purified by Con A−Sepharose affinity and SP-Sepharose ion exchange columns (Pharmacia) was adsorbed again on a Con A−Sepharose column equilibrated with buffer A [20% (w/v) glycerol, 0.6% CHAPS, 0.8 mg/mL PC, 50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin (pH 6.6)] supplemented with 1 mM $MnCl_2$ and $CaCl_2$. After the column was washed with buffer A supplemented with 1.5 mM methyl α-D-mannoside to remove the other contaminating pigments, iodopsin was eluted with buffer A supplemented with 200 mM methyl α-D-mannoside. The purified sample was then dialyzed against buffer C [50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin (pH 6.6)] with three exchanges of buffer every 1 h and three times every 3 h to prepare the PC liposome containing iodopsin. The sample was centrifuged, and the pellet was suspended in a 10 mM NaCl solution. To prepare iodopsin•NO_3 and iodopsin•free, the purified iodopsin sample was dialyzed against buffer C containing 140 mM $NaNO_3$ or no salt instead of NaCl and then centrifuged. The pellets were suspended in a 10 mM $NaNO_3$ solution or distilled water. The suspensions were then applied to a BaF_2 window (18 mm, OYO-KOKEN), followed by drying at 4 °C overnight. About 1 μL of either H_2O, D_2O, or $H_2{}^{18}O$ was put beside the film for humidification, sealed by another BaF_2 window with the aid of silicon rubber O-ring, and placed in a brass cell holder. The extent of sample hydration was monitored by the absorbance of the O−H (or O−D) stretching region of the IR absorption spectrum. We measured all the FTIR difference spectra under conditions in which the absorbances of the O−H region (3400 cm^{-1}) were in the range of 0.7−0.8. Under these conditions, about 1000 water molecules per molecule of iodopsin are present in the samples, assuming that the extinction coefficient of the O−H stretching mode of the water molecule at 3500 cm^{-1} is 100 (*12*).

Spectrophotometry. UV−vis absorption spectra were recorded with a Shimadzu model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. The system for the recording of absorption spectra was reported previously (*9*, *13*). FTIR spectra were recorded by a Bio-Rad FTS-60A/896 spectrometer according to the methods described previously (*14*). An Oxford model DN-1704 cryostat was used to cool the sample. The temperature of the sample was regulated to within 0.1 °C with a temperature controller (ITC-4, Oxford). The sample was irradiated with light from a 1 kW tungsten halogen lamp (Rikagaku Seiki) which had been passed through a glass cutoff filter (IR76 and VR68, Toshiba) or an interference filter (501 nm, Nihonshinku; 780 or 700 nm, Toshiba). The conditions of irradiation to obtain the difference FTIR spectra in iodospin•Cl and iodopsin•NO_3 systems were identical with those reported previously (*9*), while those in iodopsin•free are described in Results. For each measurement, 128 interferograms were accumulated. The difference FTIR spectra shown in the figures are the averages of 8−16 measurements.

RESULTS

Visible and Infrared Absorption Spectra of Iodopsin under Various Conditions. Figure 2a shows visible absorption spectra of iodopsin•Cl (curve 1, dashed line), iodopsin•free

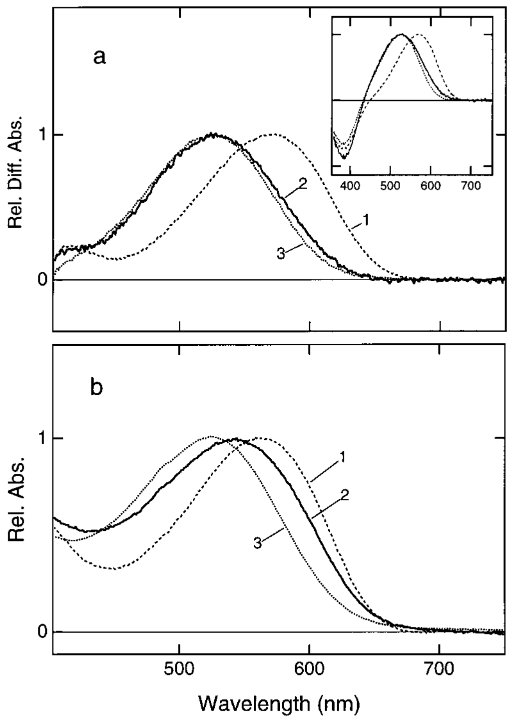

FIGURE 2: Visible absorption spectra of iodopsin•Cl (curve 1, dashed line), iodopsin•free (curve 2, solid line), and iodopsin•NO_3 (curve 3, dotted line). (a) Difference spectra of the iodopsin samples in the PC liposome suspension are calculated from the spectra recorded before and after irradiation with >480 nm light at 4 °C in the presence of 10 mM hydroxylamine. The absorption maxima (λ_{max}) of iodopsin•Cl, iodopsin•free, and iodopsin•NO_3 are 571, 528, and 526 nm, respectively. The inset shows the difference spectra calculated from the spectra before and after irradiation with >480 nm light at 4 °C in the absence of hydroxylamine. (b) Absorption spectra of the hydrated films of iodopsin at room temperature. The λ_{max} values of iodopsin•Cl and iodopsin•NO_3 are 568 and 525 nm, respectively. The blue shift by ~2 nm from those of the liposome suspension is reproduced among the different film preparations. In contrast, the λ_{max} of iodopsin•free is located at a wavelength between 540 and 570 nm, depending on the film preparations. In the figure, the λ_{max} is at 544 nm.

(curve 2, solid line), and iodopsin•NO_3 (curve 3, dotted line) in the PC liposome suspension. The difference spectra shown in the inset were calculated from the spectra recorded before and after irradiation with >480 nm light at 4 °C. The absorption maxima of iodopsin•Cl, iodopsin•NO_3, and iodopsin•free were located at 573, 528, and 528 nm, respectively. Thus, iodopsin•free exhibited an absorption maximum similar to that of iodopsin•NO_3, although it exhibited a relatively large absorbance at wavelengths longer than the maximum as compared with iodopsin•NO_3. Because the photoproduct produced by the irradiation of iodopsin displays some absorbances at wavelengths shorter than the maximum, hydroxylamine was applied to the sample to hydrolyze the retinal Schiff base of the photoproducts. The resulting retinal oxime had an absorption maximum shorter than that of the photoproducts, and therefore, the application of hydroxylamine made it possible to calculate the maximum more accurately. We added neutralized solutions of the HCl and HNO_3 forms of hydroxylamine into the iodopsin•Cl and iodopsin•NO_3 samples, respectively. In the case of the iodopsin•free sample, we added a neutralized solution of the H_2SO_4 form of hydroxylamine, because the previous report indicated that sulfate ion did not bind to the chloride binding site (*6*). The resulting spectra exhibited absorption maxima at 571, 526, and 528 nm in iodopsin•Cl, iodopsin•NO_3, and iodopsin•free samples, respectively. It should be noted that the spectral shape of iodopsin•free at wavelengths longer than the maximum scarcely changed upon addition of the H_2SO_4 form of hydroxylamine, which confirms the previous observation (*6*).

To obtain the accurate infrared spectra, water contents of the samples have to be significantly reduced. Thus, we next prepared the film samples of these pigments upon drying the PC liposome suspension on the optical windows and then hydrated them by addition of a small amount of water on the film samples, as described for iodopsin•Cl and iodopsin•NO_3 (*9*). Figure 2b shows the visible absorption spectra of the hydrated films at room temperature. Although these spectra are somewhat distorted due to the increased level of scattering at shorter wavelengths, it is clear that the absorption spectra of iodopsin•Cl and iodopsin•NO_3 are similar to those observed in the PC liposome suspension. On the other hand, the spectrum of iodopsin•free is considerably different from that observed in the PC liposome suspension. That is, the maximum shifted to 544 nm, which was 16 nm longer than that in the PC liposome. We found that iodopsin•free in the hydrated film exhibited a maximum at a wavelength between 540 and 570 nm depending on the sample preparations, although in all the preparations the 528 nm maximum was observed in the PC liposome suspension. These results suggested that a certain amount of chloride was still present in the iodopsin•free sample and that the concentration of chloride could increase to reach the constant for binding of chloride to iodopsin ($K_d = 0.1$ mM; *8*) in the course of film preparation. Therefore, a certain amount of the iodopsin•Cl state could be present in the iodopsin•free film sample. If it is the case, a mixture of iodopsin•free and iodopsin•Cl can be optically separated by selective irradiation. Thus, we tried to separate the two states by low-temperature spectroscopy.

Figure 3 shows the optical separation of iodopsin•free and iodopsin•Cl in the hydrated film of the iodopsin•free sample at 77 K. The film (curve 1 in Figure 3a) was first irradiated with 501 nm light to produce a photo-steady-state mixture containing mainly bathoiodopsin (curve 2 in Figure 3a). The sample was then irradiated with 780 nm light (curve 3 in Figure 3a)[2] and, subsequently, irradiated with 700 nm light. The difference spectra calculated from the spectra before and after the respective irradiations are shown in Figure 3b. The difference spectrum that was calculated from the spectra recorded before and after the irradiation with 780 nm light (curve 2′ in Figure 3b) exhibited positive and negative maxima at 548 and 646 nm, respectively. This spectral feature is very close to that observed in the conversion of bathoiodopsin to iodopsin in iodopsin•Cl (dotted curve in Figure 3b), suggesting that the difference spectrum corresponds to the photoreaction of iodopsin•Cl contained in the

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[2] We tried to selectively irradiate the bathoiodopsin•Cl possibly contained in the irradiated iodopsin•free sample. Because the bathoiodopsin•Cl was thought to have the most red-shifted spectrum among the products present in the sample, we tested lights of various wavelengths that caused only the photoreaction of bathoiodopsin•Cl under the criteria in which the repeated short irradiation (5−10 s) formed an isosbestic point (*15*). As a result, we found that the 780 nm irradiation formed the isosbestic point and provided visible and infrared difference spectra identical with those from the iodopsin•Cl sample as shown in Figures 3 and 4.

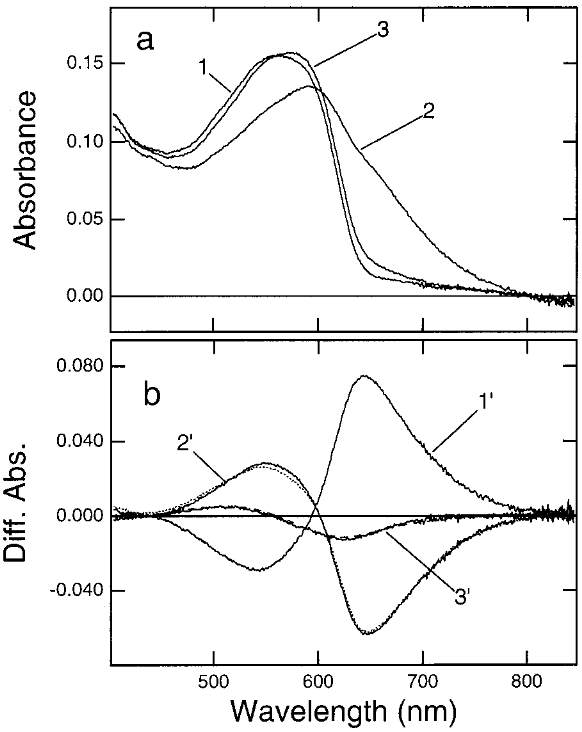

FIGURE 3: Optical separation of iodopsin•free and iodopsin•Cl in the hydrated film of iodopsin•free at 77 K. (a) The hydrated film at 77 K (curve 1) was irradiated with 501 nm light to produce a photo-steady-state mixture which dominantly contains bathoiodopsin (curve 2). The film was then irradiated with 780 nm light to revert bathoiodopsin•Cl to iodopsin•Cl (curve 3). Finally, residual bathoiodopsin•free which did not change upon irradiation with 780 nm light was converted to the original state by irradiation with 700 nm light (curve 1). (b) Difference absorption spectra calculated from the spectra recorded after irradiations with 501 (curve 1′, curve 2 − curve 1), 780 (curve 2′, curve 3 − curve 2), and 700 nm light (curve 3′, curve 1 − curve 3). The dotted and broken lines, which can be superimposed on curves 2′ and 3′, respectively, represent the difference spectrum between iodopsin•Cl and bathoiodopsin•Cl and that between iodopsin•NO_3 and bathoiodopsin•NO_3.

hydrated film of the iodopsin•free sample. This is also confirmed with the FTIR spectrum (see below). On the other hand, the difference spectrum obtained by irradiation of the sample with 700 nm light exhibits positive and negative peaks at 512 and 624 nm, respectively (curve 3′). This spectrum is likely to originate from the difference between iodopsin•free and its batho intermediate. An interesting observation is that the spectrum is identical with that obtained from iodopsin•NO_3 (broken curve in Figure 3b), suggesting that the environment around the chromophore is similar to that in iodopsin•NO_3. However, some differences in the FTIR spectra were observed between iodopsin•free and iodopsin•NO_3, indicating that iodopsin•free is not the same species as iodopsin•NO_3 (see below).

We next measured the FTIR spectra of the film sample of iodopsin•free under the same irradiation conditions as for the visible absorption spectroscopy. Curve 1 in Figure 4 is the batho/iod difference FTIR spectrum from iodopsin•Cl, which was obtained by irradiation of an iodopsin•Cl film at 77 K. The difference FTIR spectrum calculated from the spectra measured before and after irradiation of the iodopsin•free film with 780 nm light could be superimposed on this spectrum (dotted curve in Figure 4), indicating that these are identical. These results strongly suggested that the iodopsin•free film contained iodopsin•Cl.[3] The difference FTIR spectrum calculated from the spectra before and after irradiation with 700 nm light (curve 2 in Figure 4) exhibits spectral features different from those of batho/iod•Cl. The positive 1504 and negative 1531 cm^{-1} bands are characteristic for the C=C stretching (ethylenic) vibrations of the batho and original pigment, which are different from those of bathoiodopsin•Cl and iodopsin•Cl (1499 and 1524 cm^{-1}, respectively). On the other hand, they are identical with those observed in iodopsin•NO_3 (curve 3 in Figure 4). Because the frequency of the C=C stretching band correlates with the absorption maximum in the visible region, these results indicated that the pigments detected by the spectra exhibit maxima that are almost the same as those of bathoiodopsin•NO_3 and iodopsin•NO_3, but at wavelengths shorter than those of bathoiodopsin•Cl and iodopsin•Cl. Below, we compare the IR spectra of the three species in detail.

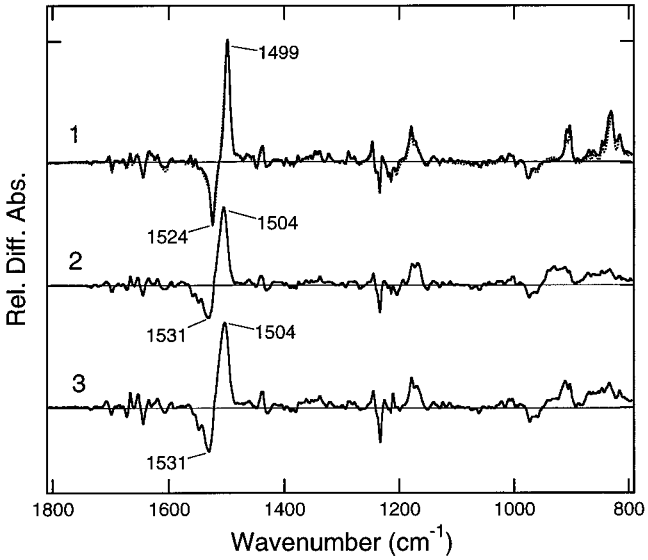

FIGURE 4: Batho/iod FTIR difference spectra of iodopsin•Cl (1), iodopsin•free (2), and iodopsin•NO_3 (3) measured at 77 K. Curves 1 and 3 are the spectra obtained from the hydrated films of iodopsin•Cl and iodopsin•NO_3 by irradiation with 710 and >670 nm light, respectively, after production of the photo-steady-state mixtures containing bathoiodopsin by irradiation with 501 nm light. The spectrum of iodopsin•Cl is the same as that previously published (*9*). The 1750−1550 and 1020−800 cm^{-1} regions of the spectrum of iodopsin•NO_3 were published previously (*9*). Curve 2 is the spectrum obtained from the hydrated film of iodopsin•free by irradiation with 700 nm light after irradiation with 780 nm light of the photo-steady-state mixture produced by irradiation with 501 nm light. The dotted line superimposed on curve 1 represents the spectrum obtained from the spectra before and after irradiation of the photo-steady-state mixture with 780 nm light. The intensities of these spectra are normalized by the 973 cm^{-1} band.

Infrared Spectral Changes in the Chromophore Vibrations among Iodopsin•Cl, Iodopsin•Free, and Iodopsin•NO_3. Figure 5 shows the batho/iod FTIR difference spectra obtained from iodopsin•Cl, iodopsin•free, and iodopsin•NO_3 in the

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[3] Because of the strong absorption of the PC liposome suspension, we were unable to measure the difference FTIR spectra not only in the water O−H region (3700−3400 cm^{-1}) but also in the amide I region (1700−1600 cm^{-1}) using the PC liposome suspensions as samples. The spectrum in the latter region provides information about the secondary structure of the protein. Therefore, a possibility that the protein structure of iodopsin•free could change upon drying is not ruled out by the experiments.

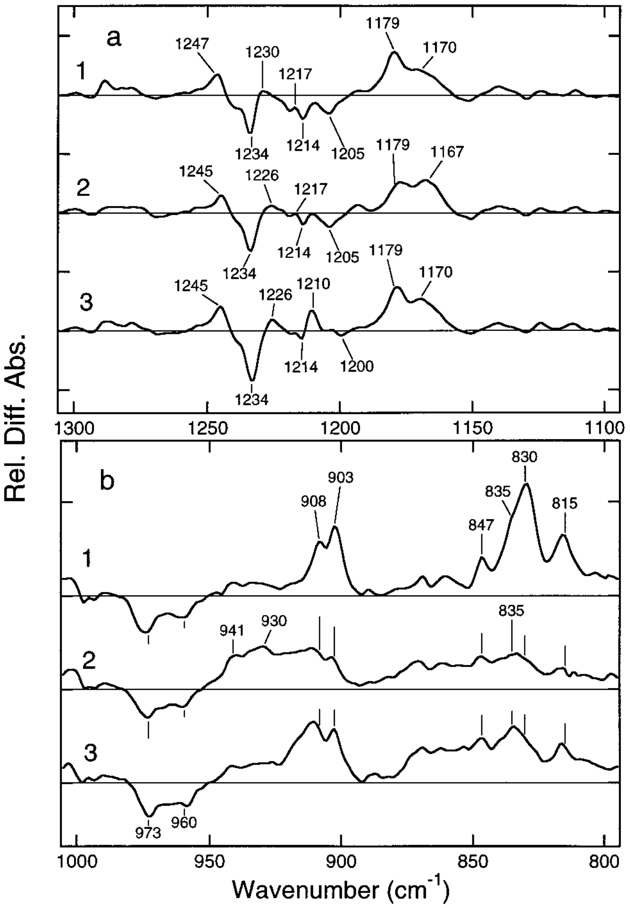

FIGURE 5: Batho/iod FTIR difference spectra of iodopsin•Cl (1), iodopsin•free (2), and iodopsin•NO_3 (3) in the region from 1300 to 1100 cm^{-1} (a) and that from 1000 to 800 cm^{-1} (b). Each spectrum was taken from Figure 4.

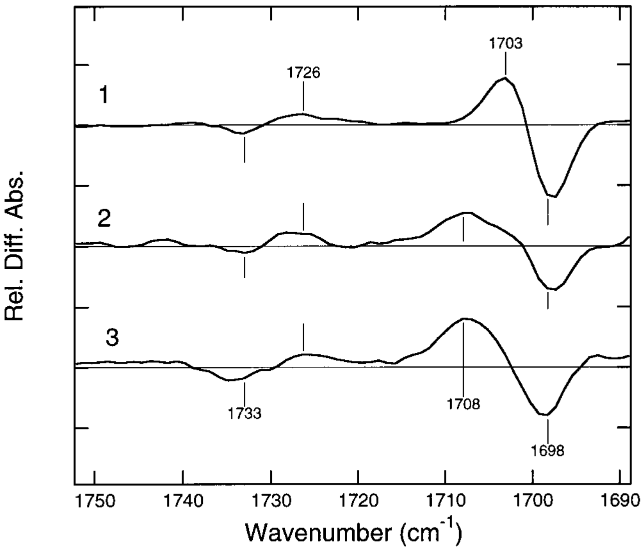

FIGURE 6: Batho/iod FTIR difference spectra of iodopsin•Cl (1), iodopsin•free (2), and iodopsin•NO_3 (3) in the 1750−1690 cm^{-1} region. Each spectrum is taken from Figure 4.

1300−1100 and 1000−800 cm^{-1} regions, where the C−C stretching and HOOP vibrations of the retinal chromophores, respectively, appear. The spectral features at the negative side due to the vibrations from the original pigment (iodopsin) look very similar, while those at the positive side are relatively different. The negative 1234 and 1214 cm^{-1} bands that were assigned to the C_{12}−C_{13} and C_8−C_9 stretching vibrations of iodopsin•Cl by resonance Raman spectroscopy (*16*) also appear in iodopsin•free and iodopsin•NO_3. Similarly, the 973 and 960 cm^{-1} bands of iodopsin•Cl which were assigned to the combination bands of the $C_{11}H$, $C_{12}H$, and $C_{14}H$ wagging modes (*9*, *16*) are also seen in the spectra of iodopsin•free and iodopsin•NO_3. The spectral features of bathoiodopsin (positive sides) in the fingerprint region are not very different, but those in the HOOP region are significantly different. The intensity of the most prominent 830 cm^{-1} band of bathoiodopsin•Cl, which contains the $C_{14}H$ wagging mode (*9*), is greatly diminished in the spectra of bathoiodopsin•free and bathoiodopsin•NO_3. The 903 cm^{-1} band assigned to the $C_{11}H$ wagging mode does not change, but the 908 cm^{-1} band assigned to the $C_{14}H$ wagging mode shifted to the higher wavenumber when chloride was depleted or substituted for nitrate. The bathoiodopsin•free displays specific bands at 941 and 930 cm^{-1}. These bands were not yet uniquely assigned, but they contained the $C_{11}H$ and/or $C_{12}H$ wagging modes, because replacement of retinal with 11,12-D_2-retinal resulted in the disappearance of these bands (data not shown). Although the spectral shape of the batho/iod•free spectrum is certainly different from that of batho/iod•NO_3, these are relatively similar compared with that of batho/iod•Cl. This tendency is also seen in the amide I region (Figure 4) and the high-frequency region (see below).

Infrared Spectral Changes in the Protein Vibrations among Iodopsin•Cl, Iodopsin•Free, and Iodopsin•NO_3. The observed anion effect on the chromophore bands in batho state is presumably accompanied by structural changes on the protein side. We thereby examined the anion effects on the protein vibrations such as protonated carboxylates, peptide carbonyls, and bound waters. Figure 6 shows the batho/iod FTIR difference spectra of iodopsin•Cl (curve 1), iodopsin•free (curve 2), and iodopsin•NO_3 (curve 3) in the 1750−1690 cm^{-1} region. A pair of positive and negative bands at 1726 (+)/1733 (−) cm^{-1} of iodopsin•Cl probably originates from the C═O stretching vibration of the protonated carboxylate (−COOH) because it shifts to 1714 (+)/1719 (−) cm^{-1} upon hydration with D_2O (data not shown). These bands did not shift upon anion substitution, indicating that the carboxylic acid is not involved in the anion binding domain. The D_2O-insensitive 1703 (+)/1698 cm^{-1} (−) bands of iodopsin•Cl are likely due to the carbonyl C═O stretching vibration of the peptide bond. Although the negative 1698 cm^{-1} band is not changed, the positive 1703 cm^{-1} band shifts to 1708 cm^{-1} in the spectra of iodopsin•NO_3 and iodopsin•free. The anion effect of the positive 1703 cm^{-1} band probably corresponds to those of the chromophore vibrations in the batho state. Therefore, the binding of chloride changes the protein conformation, thereby resulting in perturbation of the structure of the photoisomerized chromophore in the batho state.

Figure 7 shows the batho/iod FTIR difference spectra of iodopsin•Cl (curve 1), iodopsin•free (curve 2), and iodopsin•NO_3 (curve 3) in the 3700−3400 cm^{-1} region. The O−H stretching vibration of water molecules can be identified by hydrating the film sample with $H_2{}^{18}O$ so that the band is downshifted by ~10 cm^{-1}. In the spectrum of iodopsin•Cl, only the positive 3485 cm^{-1} band and the negative 3430 cm^{-1} band are insensitive to D_2O substitution (not shown). All other D_2O-sensitive positive bands at 3615, 3585, 3538, and

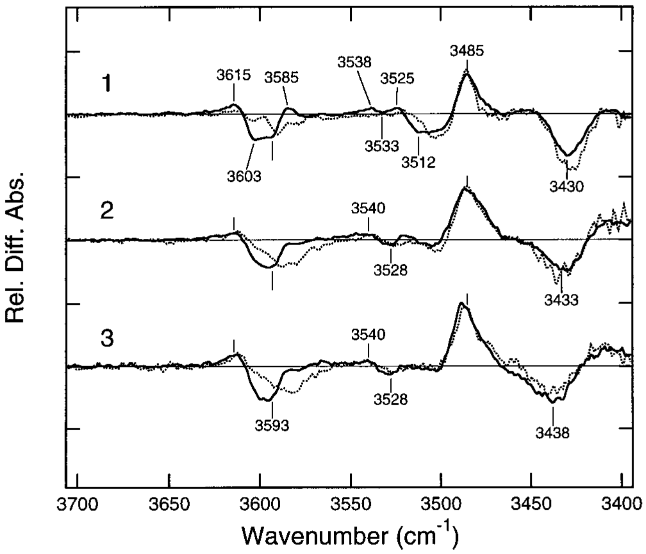

FIGURE 7: Batho/iod FTIR difference spectra of iodopsin•Cl (1), iodopsin•free (2), and iodopsin•NO$_3$ (3) in the 3700−3400 cm^{-1} region. Solid and dotted lines represent the difference spectra hydrated with H_2O and $H_2{}^{18}O$, respectively.

3525 cm^{-1} and negative bands at 3603, 3593, 3533, and 3512 cm^{-1} exhibited a downshift upon hydration with $H_2{}^{18}O$, indicating that they represent the O−H stretching vibration of water molecules. Thus, the hydrogen bonds of at least four water O−Hs are changed upon cis−trans isomerization of iodopsin•Cl. Spectral features of water O−H stretching vibrations are similar in iodopsin•NO$_3$ and iodopsin•free, where two positive peaks at 3615 and 3540 cm^{-1} and two negative peaks at 3593 and 3528 cm^{-1} are downshifted upon hydration with $H_2{}^{18}O$. The bands appearing in the 3650−3550 cm^{-1} region could originate from at least two water bands in all the iodopsin samples, although the spectral feature observed in iodopsin•Cl is different from those in iodopsin•free and iodopsin•NO$_3$. The pair of positive and negative bands at 3538 (+)/3533 (−) cm^{-1} in iodopsin•Cl should correspond to the bands at 3540 (+)/3528 (−) cm^{-1} in iodopsin•free and iodopsin•NO$_3$. Thus, the pair of bands at 3525 (+)/3512 (−) cm^{-1} is unique in iodopsin•Cl, and this water molecule is probably remote from the chromophore binding site when chloride is replaced with nitrate or its level is depleted.

DISCUSSION

In the study presented here, we have measured the visible and infrared spectra of iodopsin•free and its photoproducts at 77 K and compared them with those observed in iodopsin•Cl and iodopsin•NO$_3$. The results showed that, in addition to the chromophore electronic bands, the chromophore vibrational bands as well as the peptide and water bands are similar in iodopsin•free and iodopsin•NO$_3$ systems, but they are different from those of the iodopsin•Cl system. The differences are more prominent in the batho state, where the 11-cis chromophore is isomerized to the all-trans form. These results indicated that the binding of chloride causes a unique change in the protein conformation in iodopsin. Thus, the comparisons of the spectra of iodopsin•free with those of iodopsin•Cl and iodopsin•NO$_3$ elucidate the unique mechanism of chloride binding to iodopsin.

Mechanism of Anion Binding to Iodopsin. After the discovery of the chloride effect on the spectral properties of iodopsin and gecko visual pigment (*4*, *5*), many attempts were made to elucidate its molecular mechanism. Earlier studies showed that the binding of chloride caused an ∼40 nm red shift of the maximum (*6*, *17*) and that a divalent anion such as $SO_4{}^{2-}$ did not bind to the binding site (*6*). The binding of nitrate caused no shift in the maximum of the anion-free iodopsin (*6*), although it was later confirmed that nitrate can competitively bind to the binding site with chloride (*8*). On the other hand, the later studies on long-wavelength-sensitive pigments in the photoreceptor cells by means of microspectrophotometry showed that the binding of chloride caused a red shift in the maximum, while that of lyotropic anions such as $NO_3{}^-$, SCN^-, and $ClO_4{}^-$ caused a blue shift in the maximum from the anion-free iodopsin (*7*). The study presented here shows that iodopsin•free in PC liposomes displays a maximum at 528 nm, which is almost the same as that of iodopsin•NO$_3$. These results are consistent with the earlier work (*6*) and suggest that nitrate bound to the binding site is apart from the chromophore binding site, thereby resulting in less electrostatic interaction between nitrate and the retinal chromophore. Because the binding site is located at the putative extracellular loop (Figure 1), the binding of chloride causes rearrangement of the protein conformation which allows approach of the binding site to the chromophore binding site. This mechanism requires two conformational states of the chloride binding site and dynamic changes in the protein moiety upon binding of chloride.

While visible absorption spectra among different anions (Figure 2) originally provided the idea of the present mechanism, the FTIR spectra are consistent with the mechanism. The intensity of the HOOP band at 830 cm^{-1} which contains the $C_{14}H$ wagging mode of bathoiodopsin was much reduced in the spectra of iodopsin•free and iodopsin•NO$_3$, suggesting that the retinal polyene chain of bathoiodopsin•Cl has a specific interaction with the nearby protein. The presence of the specific interaction is also supported by the fact that the D_2O-insensitive band of bathoiodopsin•Cl at 1703 cm^{-1} was shifted in the spectra of bathoiodopsin•NO$_3$ and bathoiodopsin•free (1708 cm^{-1}, Figure 6). Thus, the microenvironment of the chromophore in bathoiodopsin•Cl is different from those of bathoiodopsin•NO$_3$ and bathoiodopsin•free, but the latter two are similar.

FTIR spectroscopy also exhibited a unique water band in the spectrum of iodopsin•Cl, which was absent in the spectra of iodopsin•NO$_3$ and iodopsin•free. This fact indicates that at least one water molecule is newly situated near the chromophore upon chloride binding. Because nitrate can bind to the same binding site as chloride does but causes no appearance of this water band, these differences should be explained by the difference in character between chloride and nitrate. Nitrate is one of the lyotropic anions whose likelihood of being hydrated 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 molecules so that the binding site tends to be connected with the chromophore binding site through a hydrogen-bonding network, including water molecules. On the other hand, binding of only a lyotropic nitrate does not produce the hydrogen-bonding network. Thus, anion binding itself is likely to occur in a simple electrostatic manner between the anion and the binding site (His194 and Lys197), while water molecules may be necessary for the constitution of the binding site at the right position.

The present spectroscopic measurements strongly suggest that some displacement of the loop region, including the chloride binding site, occurs upon chloride binding. However, the displacement may not be accompanied by a global conformational change in the protein. The amino acid residues (H194 and K197) responsible for chloride binding are situated in the loop region that is fixed at one end by helix 4 and at the other end by helix 3 through disulfide bonding between C200 and C123 (corresponding to C187 and C110 of bovine rhodopsin; *18*). The two cysteines, which form disulfide bonds, are highly conserved in many G-protein-coupled receptors (*19*). Therefore, the region including the chloride binding site may have a relatively restricted conformation so that the hydrogen-bonding network system, including water molecules, may be realized by the binding of chloride. This is also strongly suggested by the recently published rhodopsin structure (*20*; see below).

Mechanism of the Spectral Red Shift upon Chloride Binding. As has been described, iodopsin exhibits a spectral red shift upon chloride binding. In other words, a shift of the maximum can be achieved only in the presence of chloride. Several mechanisms of spectral tuning of visual pigments have been proposed: (1) a distorted structure of the chromophore (*21*), (2) the existence of a charged (*22*−*26*) or polar residue (*27*, *28*) around the chromophore, and (3) the distance between the protonated Schiff base of the chromophore and its counterion or hydrogen-bonding partner (*29*−*31*). In the fingerprint region (C−C stretching of the chromophore) of the batho/iod FTIR difference spectra, there is little spectral difference among iodopsin·Cl, iodopsin·free, and iodopsin·NO_3 (Figure 5). The HOOP bands of the original state at 973 and 960 cm^{-1} are also the same among these pigments. These results suggest that chloride binding to iodopsin does not perturb the chromophore structure, which is in contrast to the considerable effect appearing upon formation of bathoiodopsin as described above. Previous resonance Raman spectroscopy of iodopsin suggested that the spectral red shift results from the weakened electrostatic interaction between the retinal chromophore and its protein counterion (*16*). However, no obvious difference was observed in the C═N stretching region between iodopsin·Cl and iodopsin·NO_3 (*9*). Thus, it is likely that the effect of chloride on the spectral shift of the original iodopsin state originates from a long-range interaction probably through a hydrogen-bonding network, including water molecules. On the other hand, the perturbation of the C_{14} position of the chromophore in the photoisomerized bathoiodopsin state originates from the proximity of the C_{14} position of the chromophore to the network system, including chloride and waters.

During the revision of this paper, the three-dimensional structure of rhodopsin as determined by X-ray crystallography was published (*20*). The structure indicated that the extracellular loop forms the specific structure. That is, residues Ser186−Asp190 (β4) form an antiparallel β-sheet with residues Arg177−Glu181 (β3), and β4 covers the retinal chromophore from the extracellular side. The side chain of Glu181, which is the corresponding residue of the chloride binding site of iodopsin (His194), is situated within 6 Å of the C_{14} position of the chromophore. Therefore, chloride would bind to His194 with water molecules to form a hydrogen-bonding network system with residues constituting the chromophore binding site. Upon photoisomerization of the chromophore, the C_{14} position of the chromophore tends to be close to the chloride and/or water molecules within the distance of direct interaction. Our future research will aim to identify the amino acid residues involved in the network system.

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