Effect of Chloride on the Thermal Reverse Reaction of Intermediates of Iodopsin†

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ABSTRACT: Among the intermediates in the bleaching process of iodopsin, a chicken red-sensitive cone visual pigment, the batho and meta I intermediates (batho and meta I) formed at low temperatures revert to the original iodopsin by thermal reactions [Yoshizawa & Wald (1967) *Nature 214*, 566−571; Imamoto, Imai, Yoshizawa, & Shichida (1994) *FEBS Lett. 354*, 165−168]. In order to elucidate the relationship between Cl^- binding to iodopsin and these reverse reactions, we have prepared a sample of iodopsin whose Cl^--binding site is vacant (anion-unbound iodopsin) and compared the thermal reactions of its batho and meta I intermediates with those of Cl^--bound (native) and nitrate-bound iodopsins. The reverse reaction from batho is observed in both Cl^--bound and anion-unbound iodopsins, while the reaction from meta I is observed only in Cl^--bound iodopsin. These results indicate that Cl^- binding is indispensable for the reverse reaction from meta I, but not from batho. The reverse reaction from meta I has been further investigated as a function of Cl^- concentration, and the dissociation constant of Cl^- in meta I is estimated to be ~20 mM. This value is about 200 times larger than that of iodopsin (0.1 mM), and close to the physiological Cl^- concentration in photoreceptor cells, suggesting that Cl^- could be released from the protein moiety during the bleaching of iodopsin.

Iodopsin is a visual pigment in the chicken red-sensitive cones (Wald et al., 1955) and belongs to a family of long-wavelength-sensitive visual pigments among the four families (short, middle1, middle2, and long) of visual pigments (Okano et al., 1992b). Since iodopsin was the first cone visual pigment to be extracted in pure form from retinas, its biochemical and spectroscopic properties have been extensively studied to elucidate the functional difference between cone and rod photoreceptor cells. Like the rod visual pigment rhodopsin, iodopsin contains a 11-*cis*-retinal as its chromophore. The rate of retinal regeneration in iodopsin is more than 100 times faster than that in rhodopsin (Wald et al., 1955; Shichida et al., 1994). The physiologically active intermediate metaiodopsin II has formation and decay time constants shorter than those of metarhodopsin II (Shichida et al., 1993; Okada et al., 1994), although the photosensitivities of iodopsin and rhodopsin are quite similar (Okano et al., 1992a). These results correlate well with the functional difference between cone and rod photoreceptor cells (Schnapf & Baylor, 1986). Subsequently, the molecular properties of the other cone visual pigments, chicken green and chicken blue, have been investigated. The rapid regeneration of the original pigment and the shorter lifetime of the meta II intermediate are known to be common to all cone visual pigments (Shichida et al., 1994; Imai et al., manuscript in preparation). However, some properties observed in iodopsin are unique to this cone pigment.

On irradiation of iodopsin at liquid nitrogen temperature, the batho intermediate of iodopsin (bathoiodopsin) is produced (Yoshizawa & Wald, 1967). However, unlike the batho produced from rhodopsin, bathoiodopsin thermally reverts to the original iodopsin when it is warmed above liquid nitrogen temperature. The partial reversion to the original iodopsin is also observed from meta I which is produced by irradiation of iodopsin at −20 ° C (Imamoto et al., 1994). Since no reverse reactions are observed in rhodopsin and the other cone visual pigments, these reactions are unique to iodopsin. In addition, iodopsin has a Cl^--binding site in its protein moiety (Knowles, 1976; Fager & Fager, 1979; Shichida et al., 1990), and it is of interest to investigate the relationship between Cl^- binding and the thermal reversions of iodopsin intermediates.

The reverse reaction of batho has already been investigated in relation to Cl^- binding. When Cl^- in the binding site was replaced by nitrate, batho converted to the next intermediate lumiiodopsin, and no reverse reaction was observed (Imamoto et al., 1989), suggesting the possible influence of Cl^- binding during the batho to lumi transition. However, nitrate, a lyotropic anion, has physical characteristics different from Cl^-, and binding of nitrate to iodopsin as well as to other long-wavelength-sensitive visual pigments causes a large shift of the absorption maximum to a shorter wavelength (Kleinschmidt & Harosi, 1992), suggesting that nitrate may also have some unusual effect on the thermal reactions of the iodopsin intermediates. Thus, we prepared anion-unbound iodopsin and compared the thermal reactions of its intermediates with those of Cl^-- and nitrate-bound iodopsins by means of low-temperature spectroscopy. Current findings clearly show that the reverse reaction from batho to the original iodopsin is independent of Cl^- binding, while that from meta I is dependent on it. Furthermore, the efficiency of the reverse reaction of meta I has been investigated as a function of Cl^- concentration, and the dissociation constant of meta I is estimated to be about 200 times larger than that of the original iodopsin. From these results, a possible release of Cl^- from the protein moiety is implicated during the bleaching of iodopsin.

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MATERIALS AND METHODS

Sample Preparation. Iodopsin was extracted from about 2000 chicken retinas with 0.75% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)[1] in buffer E [50 mM HEPES, 140 mM NaCl, 1 mg/mL PC, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 kallikrein inhibitor units/mL aprotinin, pH 6.6 at 4 °C] and purified in the Cl^--bound form in buffer D20 [buffer E containing 0.6% CHAPS and 0.8 mg/mL PC, and supplemented with 20% (w/v) glycerol] by means of concanavalin A−Sepharose followed by CM-Sepharose column chromatographies (Okano et al., 1989, 1992b). To prepare nitrate-bound and anion-unbound iodopsins, the purified iodopsin sample was dialyzed 6 times against 80 times excess volume of buffer D20 containing 140 mM $NaNO_3$ or 140 mM sodium gluconate instead of NaCl. Finally, an equal volume of glycerol was added to each sample for spectral measurements at −20 °C, and 2 times volume of glycerol was added for measurements at liquid nitrogen temperature.

Spectroscopy. The absorption spectra were recorded on a Shimadzu MPS-2000 recording spectrophotometer interfaced to a personal computer (NEC PC9801RA). An optical cryostat (Oxford, CF1204) was used to study the photobleaching process at temperatures below 0 °C. The sample temperature was regulated to within 0.1 °C by a temperature controller (Oxford, ITC4) attached to the cryostat. To maintain the sample temperature to within 0.1 °C in the experiments shown in Figures 1, 2, and 3, a sample cell holder (Hitachi) connected to a circulator (NESLAB, RTE-210) was used. The sample was irradiated with light from a 1-kW tungsten−halogen lamp (Rikagaku-Seiki). The wavelength of the irradiation light was selected with a glass cutoff filter (Toshiba). A 5-cm water layer was placed in front of the light source to remove heat from the irradiation light.

RESULTS

Preparation of Samples. We first dialyzed Cl^--bound iodopsin against a buffer containing no salt to prepare a sample of iodopsin whose Cl^--binding site is vacant. We were able to obtain a Cl^--unbound iodopsin, but about half of the iodopsin population denatured during this manipulation. Therefore, we next dialyzed the iodopsin sample against a buffer containing 140 mM sodium gluconate instead of 140 mM NaCl and found that more than 70% of iodopsin remained stable. Because gluconate has a large ion radius, we expected that gluconate would not enter the Cl^--binding site of iodopsin so that an "anion-unbound iodopsin" could be prepared. Indeed, the absorption spectrum of iodopsin in the buffer containing 140 mM sodium gluconate is identical in shape to that in the buffer containing no salt (data not shown). Furthermore, the experimental results described below indicate that gluconate does not bind to iodopsin. Thus, the iodopsin in sodium gluconate is hereafter referred to as "anion-unbound iodopsin". Figure 1 shows the absorption spectra of Cl^--bound (native), nitrate-bound, and anion-unbound iodopsins. Their λ_{max} values are 570, 530, and 555 nm, respectively.

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PC, L-α-phosphatidylcholine from egg yolk; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

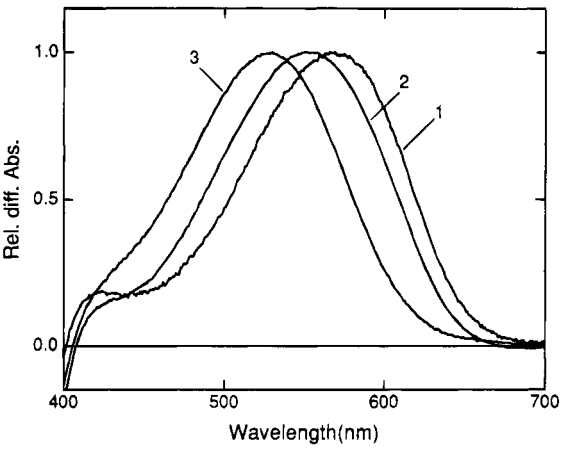

FIGURE 1: Absorption spectra of Cl^--bound iodopsin (curve 1), anion-unbound iodopsin (curve 2), and nitrate-bound iodopsin (curve 3) at 0 °C. The base line in each spectrum is the spectrum recorded after complete bleaching of the iodopsin in the presence of 10 mM NH_2OH. The spectra have been normalized at the absorption maxima. The absorption maxima of curves 1, 2, and 3 are 570, 555, and 530 nm, respectively.

Figure 2a shows the changes seen in the absorption spectrum of anion-unbound iodopsin with stepwise addition of NaCl to the sample. The absorption spectrum shifts to longer wavelength with simultaneous increase of the absorbance as the Cl^- concentration is increased. The maximum of the final spectrum is 570 nm, which is identical with that of Cl^--bound iodopsin. The increase in absorbance at 580 nm due to the formation of Cl^--bound iodopsin has been plotted as a function of Cl^- concentration (Figure 2b). From this figure, the dissociation constant of Cl^- in anion-unbound iodopsin is estimated to be about 0.1 mM. The value is identical to that obtained from the iodopsin sample in the buffer containing no salt (data not shown). Thus, we conclude that gluconate does not bind to the Cl^--binding site of iodopsin.

Similar experiments were performed using nitrate-bound iodopsin sample in which the concentration of $NaNO_3$ was 75 mM. The apparent dissociation constant of Cl^- is estimated to be 10.1 mM (Figure 2b). The large dissociation constant of Cl^- obtained in nitrate-bound iodopsin clearly shows that nitrate binds to iodopsin and affects the dissociation constant of Cl^-. If nitrate and Cl^- compete for the same binding site of iodopsin, the apparent dissociation constant should equal $K_d(1 + [NO_3]/K_n)$, where K_d and K_n are the dissociation constants for Cl^- and nitrate, respectively, and it should correlate linearly with the concentration of nitrate in the sample. So, we measured the apparent dissociation constants of Cl^- as a function of the sample nitrate concentration (Figure 2c). The data show a clear linear relationship between the apparent dissociation constant and the nitrate concentration in the sample. The slope of the fitted line in the figure is 0.14, which is identical to the value (K_d/K_n) calculated from the dissociation constants of Cl^- and nitrate which were independently observed (K_d = 0.1 mM and K_n = 0.7 mM). Therefore, we conclude that nitrate and Cl^- anions compete for the same binding site of iodopsin.

Because glycerol is added to the iodopsin sample used for low-temperature spectroscopy, we next investigated the effect of glycerol on the dissociation constant of anion-unbound iodopsin. Experiments similar to those described above were performed using a sample containing 50% (v/v) glycerol at 0 °C. The Cl^- dissociation constant of the sample

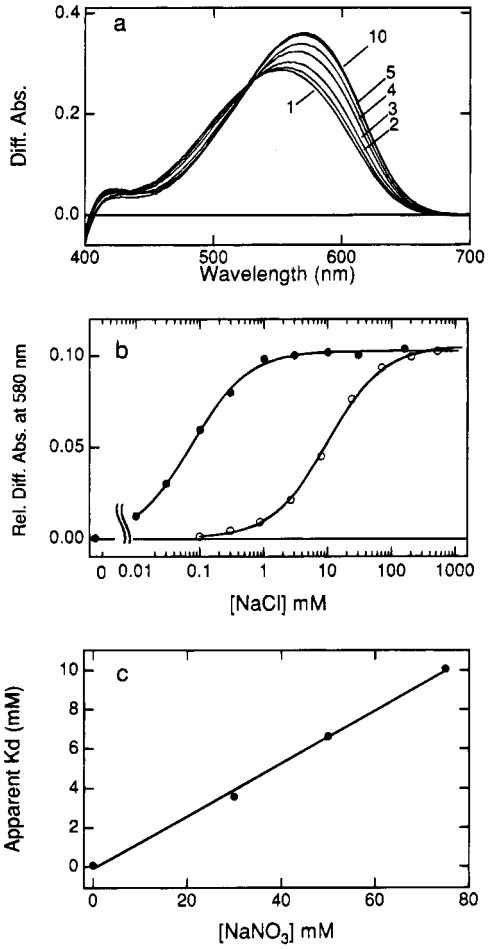

FIGURE 2: Effect of Cl^- concentration on the spectrum of anion-unbound iodopsin. (a) After recording the spectrum of anion-unbound iodopsin (curve 1), a small amount of NaCl solution was added successively to the sample, and the spectra were recorded. The concentrations of NaCl in the sample are 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 160 mM, respectively (curves 2−10). The base line is the spectrum obtained after iodopsin in the sample was completely bleached in the presence of 10 mM NH_2OH. (b) Increase in absorbance at 580 nm in (a) was plotted against Cl^- concentration of the sample (closed circle). The dissociation constant of Cl^- is estimated to be 0.1 mM from the fit of the experimental data to a curve calculated by the equation: $[I{\cdot}Cl] = [I_0][Cl^-]/(K_d + [Cl^-])$, where $[I_0]$ is the total concentration of iodopsin and K_d is the dissociation constant of Cl^-. Open circles are experimental data obtained by successive addition of NaCl solution to the nitrate-bound iodopsin sample whose $NaNO_3$ concentration is 75 mM. The dissociation constant is estimated to be 10.1 mM. (c) Apparent dissociation constants of Cl^- obtained by the addition of NaCl solution to nitrate-bound iodopsin samples whose $NaNO_3$ concentrations are 0, 30, 50, and 75 mM, respectively, are plotted as a function of nitrate concentration in the sample.

containing 50% glycerol was half that of the sample containing no glycerol, indicating that the effective concentration of NaCl in the 50% glycerol sample is 2 times higher than that in the sample without glycerol. Therefore, the NaCl concentration in the 50% glycerol sample is hereafter denoted to be twice the sample buffer concentration. It should be noted that the same dissociation constant was obtained at −10 °C, indicating that the dissociation constant is temperature-independent.

In order to obtain further evidence that gluconate does not bind to the Cl^--binding site of iodopsin, we investigated the temperature dependence of the absorption spectra of Cl^--bound, anion-unbound, and nitrate-bound iodopsins. As shown in Figure 3, the absorption maxima of Cl^--bound and

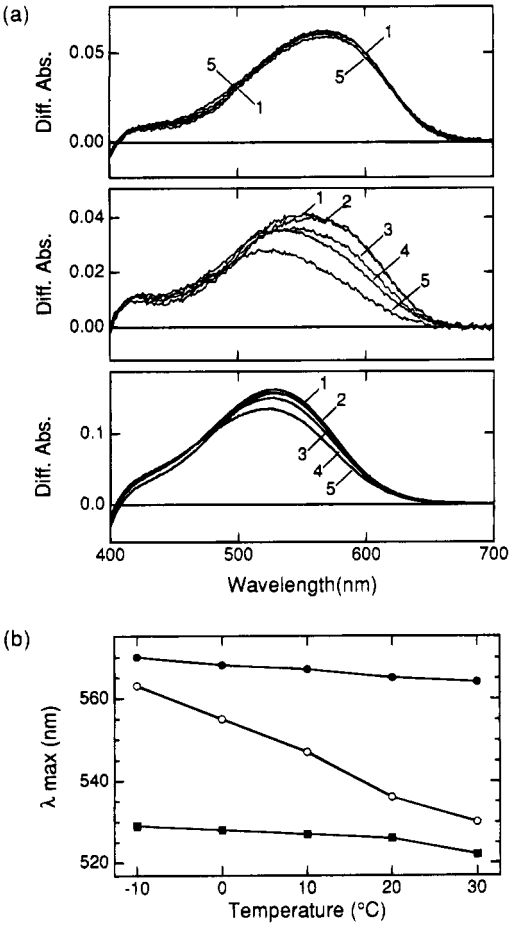

FIGURE 3: Temperature dependence of the absorption spectrum of Cl^--bound, anion-unbound, and nitrate-bound iodopsins. (a) Absorption spectra of Cl^--bound (upper panel), anion-unbound (middle panel), and nitrate-bound (lower panel) iodopsins were recorded at −10, 0, 10, 20, and 30 °C (curves 1−5). The base line of each spectrum is the spectrum obtained after each sample was completely bleached in the presence of 10 mM NH_2OH. (b) Shifts of absorption maxima in samples of Cl^--bound (closed circles), anion-unbound (open circles), and nitrate-bound (closed squares) iodopsins are plotted as a function of temperature.

nitrate-bound iodopsins shift by less than 10 nm between −10 and 30 °C, whereas the maximum of anion-unbound iodopsin varies between 530 and 565 nm. We speculate that the large change of the absorption spectrum observed in anion-unbound iodopsin reflects the flexible structure of the protein moiety due to the lack of anion binding. This flexibility may also explain the decreased stability of the anion-unbound iodopsin structure. Therefore, the temperature dependence of the absorption spectrum suggests that gluconate does not bind to the Cl^--binding site of iodopsin.

Thermal Behavior of the Batho Intermediate. To elucidate the effect of Cl^- binding on the reverse reaction from batho, we compared the thermal reactions of batho produced in anion-unbound, Cl^--bound, and nitrate-bound iodopsins. As seen in Cl^--bound and nitrate-bound iodopsins, anion-unbound iodopsin converts to batho when it is irradiated with green light (501 nm) at −195 °C. Photoreversibility among anion-unbound iodopsin, batho, and the iso pigment was also demonstrated by changing the wavelength of the irradiation light. Thus, the photochemical reactions of anion-unbound iodopsin are quite similar to those observed in Cl^--bound and nitrate-bound iodopsins. On the other hand, about 90% of anion-unbound iodopsin is recovered when the sample is warmed to 0 °C (Figure 4b). This property is the same as

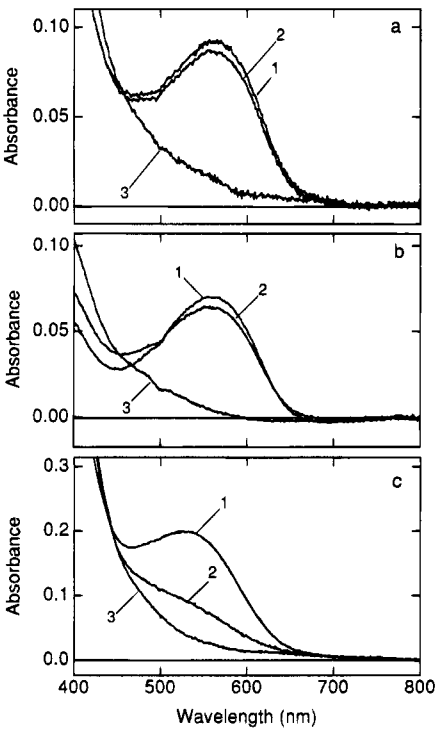

FIGURE 4: Thermal reactions of batho intermediates produced from Cl^--bound (a), anion-unbound (b), and nitrate-bound (c) iodopsins at −195 °C. After recording the spectrum at 0 °C (curves 1), each iodopsin sample was cooled to −195 °C and irradiated with green light (501 nm) until a photo-steady-state mixture was produced. Then it was warmed to 0 °C, and the spectrum was recorded (curves 2). Finally, it was irradiated with red light (>660 nm) at 0 °C to bleach the residual pigments in the sample (curves 3).

that observed in Cl^--bound iodopsin (Figure 4a), but different from that observed in nitrate-bound iodopsin (Figure 4c). Hence, we conclude that the thermal recovery of the original pigment from batho is independent of Cl^- binding, and that the absence of the reverse reaction can be ascribed solely to the effect of the nitrate ion bound to iodopsin.

Thermal Behavior of the Meta I Intermediate. To check the Cl^- effect on the thermal reaction of meta I, we irradiated Cl^--bound, anion-unbound, and nitrate-bound iodopsins at −20 °C and recorded the subsequent spectral changes in the dark (Figure 5). The conversion of meta I to meta II is observed in all three samples. However, the reverse reaction from meta I to iodopsin is only found in Cl^--bound iodopsin, where we see an increase in its absorbance at >590 nm corresponding to the recovery of iodopsin (Figure 5a). These data show that the reverse reaction from meta I to iodopsin depends on the binding of Cl^-.

Next, we examined the effect of Cl^- concentration on the reverse reaction of meta I (Figure 6). Positive absorbances above 590 nm increase proportionally with Cl^- concentration. The amount of iodopsin formed by the reverse reaction in each sample was estimated by fitting the difference spectrum at wavelengths from 620 to 650 nm with the spectrum of iodopsin (broken curves in Figure 6). Then the spectrum of iodopsin was subtracted from the difference spectrum (dotted curves in Figure 6). This subtraction enabled us to estimate the spectrum and the amount of meta I produced by irradiation of the sample at −20 °C, because meta II formed from meta I displays no absorbance at wavelengths longer than 460 nm. It should also be noted that the iodopsin spectrum was sufficient to reasonably fit the difference spectrum from 620 to 650 nm (Figure 6), indicating that negligible amounts of the lumi intermediate, which potentially could contribute to the absorbance in this region, were present in the irradiated sample under our experimental conditions.

The spectrum of meta I calculated by the subtraction procedure exhibits a maximum at longer wavelength as the Cl^- concentration is increased. This may be explained by the presence of two forms of meta I: a Cl^--bound and a Cl^--unbound form. When the spectra are normalized at the absorption maxima and superimposed, an isosbestic point between the Cl^--bound and Cl^--unbound forms of meta I is located at 490 nm. Therefore, in the following calculations, the amount of meta I was estimated from the absorbance at 490 nm in order to cancel the Cl^- effect of meta I.

The amount of iodopsin formed by the thermal reaction of meta I was estimated from the equation:

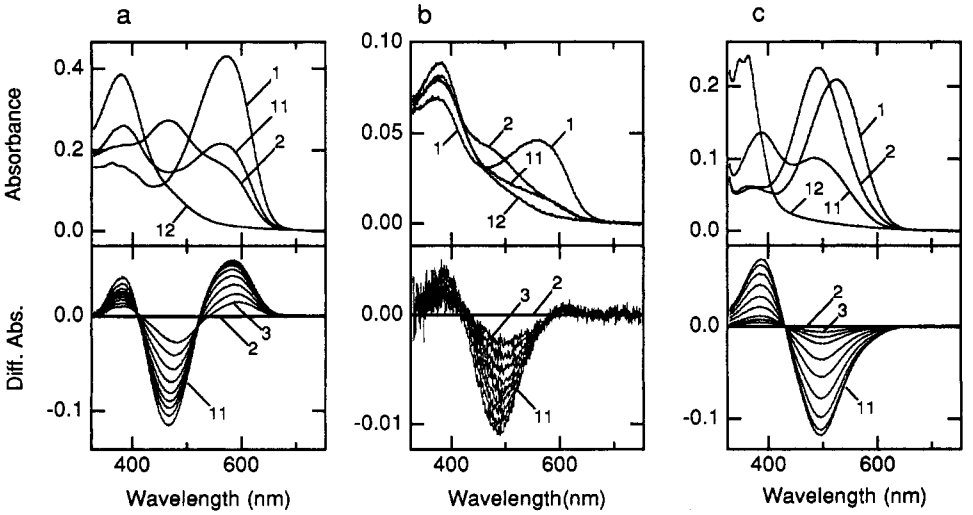

FIGURE 5: Photochemical and subsequent thermal reactions of Cl^--bound (a), anion-unbound (b), and nitrate-bound iodopsins (c) at −20 °C. Each iodopsin sample was cooled to −20 °C (curves 1) and irradiated with >590 nm light for 10 s (curves 2). Then the sample was incubated for 2.5, 5, 10, 20, 40, 80, 160, 320, and 640 min (curves 3−11). After a 640 min incubation, the sample was warmed to 0 °C and irradiated with >590 nm light for 40 min to completely bleach the residual pigments in the sample (curves 12). Nitrate-bound iodopsin sample was bleached completely in the presence of 10 mM NH_2OH. Base lines in the lower panels are the spectra recorded immediately after irradiation of the sample at −20 °C.

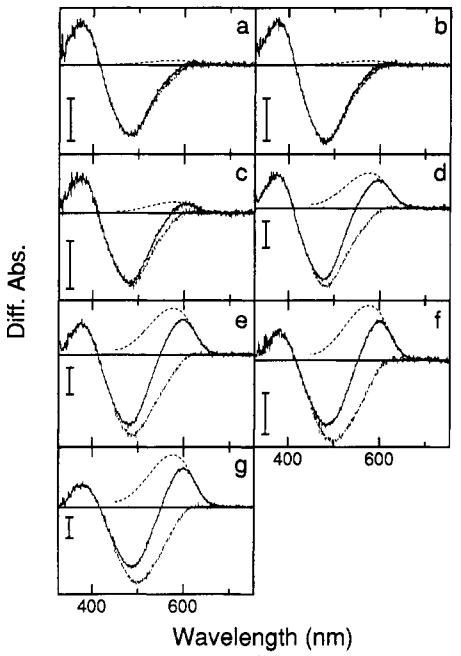

FIGURE 6: Effect of Cl^- concentration on the amount of iodopsin produced by the reverse reaction at −20 °C. In each panel, the solid curve is the difference of the spectrum recorded 2.5 min after irradiation of iodopsin with >590 nm light for 10 s and the spectrum recorded immediately after irradiation. Base lines are the spectra recorded immediately after irradiation. The broken curve is the iodopsin spectrum whose magnitude was estimated by fitting the difference spectrum between 620 and 650 nm. The dotted curve is the spectrum obtained after subtracting the iodopsin spectrum from the difference spectrum. The concentration of NaCl in each sample is 0.1 mM (a), 0.2 mM (b), 10 mM (c), 20 mM (d), 50 mM (e), 160 mM (f), or 600 mM (g), respectively. Scale bar in each figure indicates 0.1 absorbance.

$$\text{Iod/MI} = (A_{\text{Iod}}/A_{\text{MI}})(\epsilon_{\text{MI}}/\epsilon_{\text{Iod}}) \quad (1)$$

where Iod is the amount of iodopsin formed from meta I and MI is the total amount of meta I formed by irradiation of the sample at −20 °C. ϵ_{Iod} and ϵ_{MI} are the extinction coefficients of iodopsin at 570 nm and meta I at 490 nm, respectively. A_{Iod} is the absorbance of the spectrum of recovered iodopsin at 570 nm estimated from the fitting procedure (broken curves in Figure 6), and A_{MI} is the absorbance of the meta I spectrum at 490 nm obtained after subtraction of the iodopsin spectrum (dotted curves in Figure 6). Since ϵ_{Iod} and ϵ_{MI} are the fixed values, we are able to estimate the amount of iodopsin relative to the total amount of meta I. The relative amount of iodopsin is plotted as a function of Cl^- concentration in the sample in Figure 7.

Since the reverse reaction from meta I depends on the Cl^- concentration, it is reasonable to speculate that the reaction takes place from only the Cl^--bound form of meta I. The dissociation constant of Cl^- in meta I was estimated to be about 20 mM by fitting the experimental data with the curve shown in Figure 7. The curve was calculated according to the equation:

$$[\text{Meta I·Cl}] = [\text{Meta I}_0] - K_d[\text{Meta I}_0]/(K_d + [\text{Cl}]) \quad (2)$$

where [Meta I·Cl] and [Meta I_0] are the concentration of Cl^--bound meta I and the total concentration of meta I, respectively.

DISCUSSION

In the present study, we have prepared an "anion-unbound iodopsin" sample that contained 140 mM sodium gluconate.

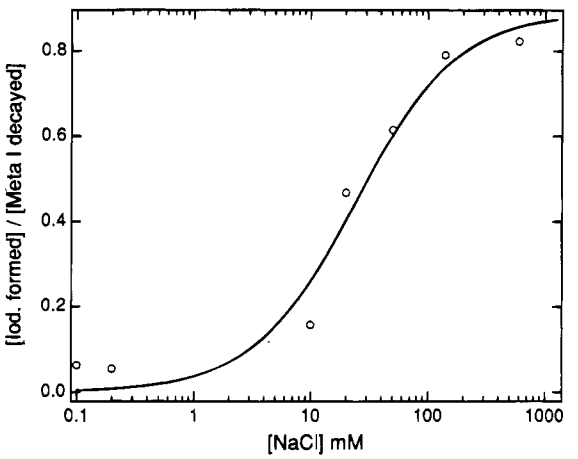

FIGURE 7: Effect of Cl^- concentration on the relative amount of iodopsin produced from the thermal reaction of meta I. The solid curve in the figure is the fit to the experimental data points. The calculation procedure is described in the text.

Iodopsin in this sample displays an absorption maximum (555 nm) and a dissociation constant of Cl^- (0.1 mM) similar to those in the sample prepared by dialysis against a buffer containing no salt, indicating that gluconate does not bind to the Cl^--binding site of iodopsin. The absorption spectrum of anion-unbound iodopsin is strongly temperature-dependent, in contrast to those of Cl^--bound and nitrate-bound iodopsins, indicating that the binding of an anion to the Cl^--binding site of iodopsin stabilizes the protein moiety of iodopsin. This is consistent with results from a previous study (Shichida et al., 1990).

We confirmed that nitrate and Cl^- compete for the same binding site as previously suggested (Imamoto et al., 1989; Shichida et al., 1990; Kleinschmidt & Harosi, 1994). The absorption maximum of nitrate-bound iodopsin is considerably blue-shifted from those of anion-unbound and Cl^--bound iodopsins, which is consistent with that observed in the native membrane of the photoreceptor cells (Kleinschmidt & Harosi, 1994), but different from that observed in 2% digitonin solution (Shichida et al., 1990). Since digitonin may alter the conformation of the protein moiety of iodopsin, we solubilized iodopsin in a mixture of CHAPS and PC where the conformation of iodopsin might be closer to that in the native photoreceptor cells (Okano et al., 1989). The blue-shifted absorption maximum of nitrate-bound iodopsin may be due to the perturbation of the hydrogen network near the Schiff base region of the chromophore (Kleinschmidt & Harosi, 1994), although the amino acid residues responsible for Cl^- binding are located near the extracellular surface of the iodopsin molecule (Wang et al., 1993).

In 1967, Yoshizawa and Wald reported that batho formed from the Cl^--bound iodopsin reverts to iodopsin when it is warmed from liquid nitrogen temperature. Later we revealed that the reverse reaction of batho does not take place in nitrate-bound iodopsin (Imamoto et al., 1989) and suggested that the reverse reaction could depend on the binding of Cl^- to iodopsin. However, the present study clearly shows that the reverse reaction also takes place in anion-unbound iodopsin, indicating no relationship between Cl^- binding and the reverse reaction from batho. The reverse reaction is apparently a result of the unique and specific conformation of the protein moiety of iodopsin. In fact, no reverse reaction is observed in Gecko visual pigment (Gecko green, P521) which also has a Cl^--binding site (Kojima et al., 1995).

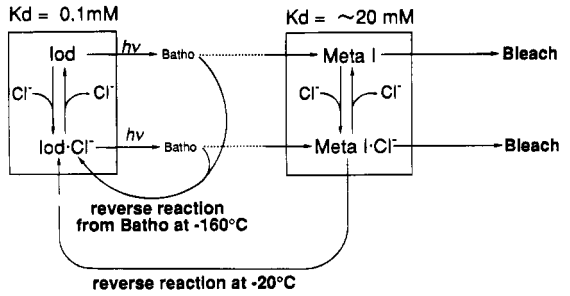

FIGURE 8: Photobleaching process of iodopsin.

On the other hand, our results demonstrate a relationship between Cl^- binding and the reverse reaction of meta I. Furthermore, meta I exists as a mixture of Cl^--bound and -unbound forms, whose proportion depends on the Cl^- concentration in the sample. Accumulated evidence on the bleaching process of rhodopsin indicates that meta I has a protein conformation around the chromophore which is highly adapted for the all-trans form, while the other regions of the protein remain in their original state (Imai et al., 1994). Since large conformational changes of the entire protein take place during the meta I to meta II transition (Matthews et al., 1963; Ostroy et al., 1974; Parkes & Liebman, 1984; Imai et al., 1994), our data indicate that Cl^- binding suppresses this large conformational change of meta I probably by perturbing the hydrogen network system in iodopsin. In other words, if meta I is in the Cl^--bound form, the configurational change of the chromophore back to the 11-cis form takes place more easily (or has a lower activation barrier) compared to the conformational change of the protein moiety to meta II, thereby resulting in the reverse reaction to iodopsin. Figure 8 shows the bleaching process of iodopsin, in which the states of Cl^- binding in both iodopsin and meta I are emphasized. Iodopsin has a Cl^- dissociation constant of about 0.1 mM, so that almost all of the iodopsin in photoreceptor cells exists in the Cl^--bound form at the physiological Cl^- concentration (12−24 mM; Kaneko & Tachibana, 1986). Upon absorption of a photon, iodopsin converts to meta I through batho. Since meta I has a dissociation constant of about 20 mM, it tends to release Cl^- from the protein moiety, and hence converts efficiently to meta II, the physiologically active intermediate of iodopsin (Okada et al., 1994). The reverse reaction of meta I to the original iodopsin, on the other hand, would occur from the Cl^--bound form of meta I.

Because the reverse reaction of meta I to iodopsin would result in a lower production of meta II, it represents a waste of photon signal and low photosensitivity. Therefore, it is of interest to investigate whether or not the reverse reaction takes place under physiological conditions. Our preliminary experiments indicate that the photosensitivity of iodopsin at 10 °C in the presence of 140 mM NaCl is identical to that in the presence of 2 mM NaCl, although the reverse reaction at −20 °C is greatly suppressed in 2 mM NaCl. These results indicate that the reverse reaction takes place only at low temperatures and that the dissociation constant of Cl^- in meta I seems to be temperature-dependent. On the other hand, the original iodopsin has a dissociation constant which is temperature-independent. Therefore, the physiological significance of the reverse reaction is not clear yet.

The reverse reaction to the original pigment at low temperature is also observed in bacteriorhodopsin (bR), where the L intermediate preferentially converts to the original bR at low temperature, but converts to the M intermediate at room temperature (Iwasa et al., 1980). Since changes in the hydrogen network system which includes water molecule(s) near the retinal Schiff base are essential for the conversion of L to M (Maeda et al., 1992), the Cl^- dissociation constant of the residue(s) in iodopsin responsible for the protein transitions might also be temperature-dependent. Taken together, our results suggest that the conformational changes of the protein moiety in iodopsin are regulated by the binding of Cl^-.

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REFERENCES

Fager, L. Y., & Fager, R. S. (1979) *Exp. Eye Res. 29*, 401−408.
Imai, H., Mizukami, T., Imamoto, Y., & Shichida, Y. (1994) *Biochemistry 33*, 14351−14357.
Imamoto, Y., Kandori, H., Okano, T., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1989) *Biochemistry 28*, 9412−9416.
Imamoto, Y., Imai, H., Yoshizawa, T., & Shichida, Y. (1994) *FEBS Lett. 354*, 165−168.
Iwasa, F. T., & Yoshizawa, T. (1980) *Biophys. Struct. Mech. 6*, 253−270.
Kaneko, A., & Tachibana, M. (1986) *J. Physiol. 373*, 443−461.
Kleinschmidt, J., & Harosi, F. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9181−9185.
Knowles, A. (1976) *Biochem. Biophys. Res. Commun. 73*, 56−62.
Kojima, D., Imai, H., Okano, T., Fukada, Y., Crescitelli, F., Yoshizawa, T., & Shichida, Y. (1995) *Biochemistry 34*, 1096−1106.
Maeda, A., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1992) *Biochemistry 31*, 462-467.
Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215−240.
Okada, T., Matsuda, T., Kandori, H., Fukada, Y., Yoshizawa, T., & Shichida, Y. (1994) *Biochemistry 33*, 4940−4946.
Okano, T., Fukada, Y., Artamonov, I. D., & Yoshizawa, T. (1989) *Biochemistry 28*, 8848−8856.
Okano, T., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1992a) *Photochem. Photobiol. 56*, 995−1001.
Okano, T., Kojima, D., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1992b) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5932−5936.
Ostroy, S. E. (1974) *Arch. Biochem. Biophys. 164*, 275−284.
Parkes, J. H., & Liebman, P. A. (1984) *Biochemistry 23*, 5054−5061.
Schnapf, J., & Baylor, D. A. (1986) *Sci. Am. 256*, 32−39.
Shichida, Y., Kato, T., Sasayama, S., Fukada, Y., & Yoshizawa, T. (1990) *Biochemistry 29*, 5843−5848.
Shichida, Y., Okada, T., Kandori, H., Fukada, Y., & Yoshizawa, T. (1993) *Biochemistry 32*, 10832−10838.
Shichida, Y., Imai, H., Imamoto, Y., Fukada, Y., & Yoshizawa, T. (1994) *Biochemistry 33*, 9040−9044.
Wald, G., Brown, P. K., & Smith, P. H. (1955) *J. Gen. Physiol. 38*, 623−681.
Wang, A. B. A., & Oprian, D. D. (1993) *Biochemistry 32*, 2125−2130.
Yoshizawa, T., & Wald, G. (1967) *Nature 214*, 556−571.

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