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Effect of Chloride Ion on the Thermal Decay Process of the Batho Intermediate of Iodopsin at Low Temperature†

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ABSTRACT: The photochemical and the subsequent thermal behaviors of iodopsin (Cl^--bound form) and *N*-iodopsin (iodopsin whose Cl^- was replaced by NO_3^-) in CHAPS-phosphatidylcholine (PC) were studied by low-temperature spectrophotometry. Irradiation of the iodopsin preparation at −185 °C produced a photo-steady-state mixture composed of iodopsin, bathoiodopsin, and isoiodopsin. Bathoiodopsin was thermally reverted to the original iodopsin. These results were almost the same as those reported previously [Yoshizawa, T., & Wald, G. (1967) *Nature 214*, 566-571] in which iodopsin was extracted with 2% digitonin. Therefore, photochemical and subsequent thermal behaviors of iodopsin were independent of the detergent to solubilize iodopsin. Irradiation of *N*-iodopsin at −185 °C produced the similar photo-steady-state mixture. However, *N*-bathoiodopsin was thermally converted to the next intermediate, presumably *N*-lumiiodopsin. These results suggest that the batho–lumi transition of iodopsin at low temperature is likely to be inhibited by the Cl^- bound to the protein moiety of iodopsin, while at room temperature the Cl^- bound to iodopsin could be released on the conversion process of batho- to lumiiodopsin.

Since the discovery of iodopsin (Wald et al., 1937), differences in the biochemical and spectroscopic nature between the rod pigment rhodopsin and the cone pigment iodopsin have been investigated with great interest. Although the difficulty of purification of cone pigments prevented their precise characterization, the following unique natures of iodopsin have been elucidated.

Like rhodopsin, iodopsin has an 11-*cis*-retinal as its chromophore, but there are distinct differences owing to difference in the protein moiety between them (Wald et al., 1955; Matsumoto et al., 1975). One of the most prominent differences in the primary photochemical process is that bathoiodopsin produced by irradiation of iodopsin at liquid nitrogen temperature is thermally reverted to the original iodopsin, while bathorhodopsin bleaches to *all-trans*-retinal and opsin through

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several intermediates (Yoshizawa & Wald, 1967).

Recently, we have succeeded in purifying iodopsin using a mixture of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)[1] and phosphatidylcholine (PC) as one of the solubilizers. The purified iodopsin thus obtained displays an absorption maximum at 571 nm, which is very close to that obtained by microspectrophotometry (569 nm; Bowmaker & Knowles, 1977) or that measured in the membrane suspension (572 nm; Fukada & Yoshizawa, 1982), but is different from that of the digitonin extract (562 nm). Since the CHAPS–PC extract seems to be more close to the native form than the digitonin extract, it is of interest to examine, using the CHAPS–PC extract, whether or not the thermal reversion of the batho intermediate is due to the artifact caused by solubilization of iodopsin with digitonin. The present work confirmed the bathoiodopsin in the CHAPS–PC extract was also reverted to iodopsin at low temperatures.

On the other hand, iodopsin has chloride binding site(s) in its protein moiety; removal of Cl^- from iodopsin causes a blue-shift of the absorption spectrum (chloride effect), while rhodopsin exhibits no chloride effect (Knowles, 1976; Fager & Fager, 1979). Addition of a large excess of NO_3^- over Cl^- in an iodopsin–2% digitonin extract displays the similar blue-shift of the absorption spectrum of iodopsin (Kato et al., 1984). In order to get a clue to the relationship between the thermal reversion of bathoiodopsin and the Cl^- binding to iodopsin, the photochemical and the subsequent thermal reactions of CHAPS–PC-solubilized iodopsin whose Cl^- binding site(s) was (were) occupied by NO_3^- (*N*-iodopsin) were also studied. The present experiments demonstrate that a bathochromic product formed by irradiation of *N*-iodopsin at liquid nitrogen temperature bleaches on warming like bathorhodopsin.

Materials and Methods

Purification of Iodopsin. Iodopsin was purified from chicken retinas in a CHAPS–PC preparation according to a method recently developed (Okano et al., 1989). Briefly, a mixture of cone and rod outer segments was isolated from fresh chicken retinas by means of a 40% (w/v) sucrose flotation method in a standard buffer [50 mM HEPES, 140 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 4 μg/mL leupeptin, 50 kallikrein inhibitor units/mL aprotinin, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 6.6] under dim red light (>660 nm). Since the outer segments thus obtained contained some bleached products of iodopsin, they were regenerated by addition of 2 times molar excess of 11-*cis* retinal. Then the visual pigments were extracted with 0.75% CHAPS in the standard buffer containing 1 mg/mL PC. This extract (4 volumes) was diluted with the standard buffer (1 volume) to decrease the concentration of CHAPS and PC to 0.6% and 0.8 mg/mL, respectively. Iodopsin in the extract was then adsorbed to a concanavalin A–Sepharose affinity column, followed by elution with 1.5 mM methyl α-D-mannoside in the elution buffer (standard buffer supplemented with 0.6% CHAPS and 0.8 mg/mL PC). The fractions which mainly contained iodopsin (more than 90% of the total visual pigment) were collected, followed by addition of glycerol at a final concentration of 20% (w/v) to keep iodopsin stable. Then they were concentrated about 100 times with an ultrafiltration membrane (AMICON, YM30). For low-temperature spectrophotometry, glycerol was added to the concentrated preparation to give a final concentration of 71% (v/v). Hydroxylamine was not added to this preparation, because it gradually bleached iodopsin in the dark at room temperature.

Preparation of N-Iodopsin. Since iodopsin whose Cl^- binding site(s) was (were) vacant was easily denatured (Kato et al., 1984), the Cl^- binding site(s) was (were) occupied by NO_3^- as follows. Iodopsin in Cl^--bound form prepared as described above (without addition of 71% glycerol) was dialyzed against the elution buffer without NaCl for 3 days in the dark with several renewals of the buffer, followed by gradual addition of the buffer supplemented with 3 M $NaNO_3$ until the absorption maximum displayed no further blue-shift (final concentration 630 mM). For low-temperature spectrophotometry, the prepartion was concentrated by the ultrafiltration membrane and then mixed with glycerol to give a final concentration of 53% (v/v). No hydroxylamine was added to this preparation.

Spectrophotometry. All the spectra were recorded with a Shimadzu Model MPS-2000 spectrophotometer interfaced with an NEC PC-9801F computer. A glass optical cryostat (Yoshizawa & Shichida, 1982) for keeping the sample at low temperature was placed in the sample compartment of the spectrophotometer. The temperature of the sample was controlled by dropping liquid nitrogen into the liquid nitrogen reservoir of the cryostat and monitored by a copper–constantan thermocouple. To correct the scattering of the measuring light caused by cracks in the sample on cooling, opal glasses were placed in both sample and reference sides of the spectrophotometer (Yoshizawa & Shichida, 1982). The sample was irradiated with light from a 1-kW tungsten–halogen lamp (Sanko) which had passed through a combination of a glass cutoff filter (VR63, VR61, or V059; Toshiba), a band-pass filter (B-48S; Toshiba), and an interference filter (547 or 501 nm; Nihonshinku). To obtain a required intensity of light, a neutral density filter was used (TND25; Toshiba). A 5-cm water layer was placed in front of the light source to remove heat radiation contained in the irradiation light.

Results

Iodopsin. The experiments were started to clarify whether iodopsin in the CHAPS–PC preparation presented similar photochemical behavior as that in the digitonin preparation (Figure 1).

An absorption spectrum of the iodopsin preparation measured at 0 °C is shown in Figure 1a (curve 1). The inset in Figure 1a shows the difference spectrum of the iodopsin preparation before and after extensive irradiation with a red light (>590 nm) at 0 °C (curves 1 and 19 in Figure 1a). Its absorption maximum was located at 571 nm, which was a 9-nm longer wavelength than that observed in the 2% digitonin preparation (562 nm; Wald et al., 1955). The value obtained in the CHAPS–PC preparation was very close to that measured in the membrane (572 nm; Fukada & Yoshizawa, 1982).

On cooling the sample to −185 from 0 °C, the spectrum was sharpened and shifted the absorption maximum to 590 nm (curve 2 in Figure 1b). Irradiation of the sample at this temperature with a green light (547 nm) caused a gradual red-shift in absorption spectra, resulting in formation of bathoiodopsin (curves 3–8). A prolonged irradiation produced a photo-steady-state mixture whose spectrum displayed no further change by the irradiation (curves 7 and 8).

The photoreversibility among iodopsin, bathoiodopsin, and isoiodopsin at −185 °C was shown in Figure 1c. Namely, the irradiation of iodopsin (curve 2) with the green light at −185

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PC, L-α-phosphatidylcholine from fresh egg yolk; λ_{max}, absorption maximum in the visible region.

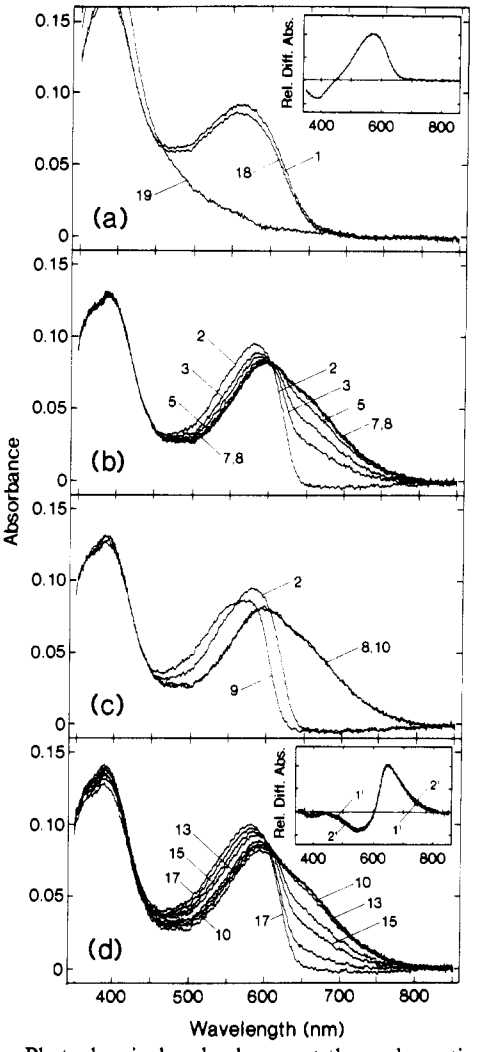

FIGURE 1: Photochemical and subsequent thermal reactions of iodopsin. (a) Absorption spectra of iodopsin and its bleaching products at 0 °C. The spectrum of the iodopsin–71% glycerol mixture was measured at 0 °C (curve 1), and then it was cooled to −185 °C [curve 2 in (b)], followed by a series of irradiations with a different color light shown in (b) and (c). Then the photo-steady-state mixture containing mainly bathoiodopsin [curves 10 in (c) and (d)] was warmed to 0 °C after stepwise warming to −120 °C [curve 17 in (d)], followed by spectral measurement (curve 18). After extensive irradiation with red light (>590 nm) at 0 °C, the spectrum was measured (curve 19). Inset: Difference absorption spectrum of iodopsin before and after complete bleaching with red light (curves 1 and 19, λ_{max} = 571 nm). (b) Formation of a photo-steady-state mixture containing mainly bathoiodopsin at −185 °C. The iodopsin preparation (curve 1) was cooled to −185 °C (curve 2) and then irradiated with green light (547 nm) for a total of 5, 10, 20, 40, 80, and 160 s (curves 3–8, respectively) until a photo-steady-state mixture was produced. (c) Photoreversibility among iodopsin, bathoiodopsin, and isoiodopsin at −185 °C. The original iodopsin (curve 2) was irradiated with green light for 160 s until the photo-steady-state mixture was produced (curve 8). Then the sample was irradiated with red light (>610 nm) for 1280 s (curve 9) for formation of mainly isoiodopsin. This preparation was irradiated again with green light for 320 s (curve 10). It should be noted that curve 10 was in good agreement with curve 8, indicating that iodopsin, bathoiodopsin, and isoiodopsin were freely interconvertible by light at −185 °C. (d) Reversion process of bathoiodopsin to iodopsin by stepwise warming. The photo-steady-state mixture containing mainly bathoiodopsin (curve 10) was warmed in the dark in a stepwise manner to −180, −170, −160, −150, −140, −130, and −120 °C (curves 11–17, respectively). All the spectra were measured at −185 °C after being warmed to the required temperature. Inset: Difference spectra between iodopsin and bathoiodopsin [curve 1′, which was calculated by subtracting curve 2 from curve 3 in (b)] and between curves 10 and 17 in (d) (curve 2′) which were normalized at their peaks. They were in good agreement with each other. Thus, curve 17 should correspond to iodopsin, indicating that bathoiodopsin reverted to iodopsin on warming.

°C induced the photo-steady-state mixture containing mainly bathoiodopsin with relatively small amounts of iodopsin and isoiodopsin (curve 8). The subsequent irradiation with red light (>610 nm) caused formation of mainly isoiodopsin (curve 9). Reirradiation of isoiodopsin with the green light (curve 10) gave a spectrum identical with curve 8, indicating that iodopsin, bathoiodopsin, and isoiodopsin were perfectly interconvertible by light at −185 °C.

Then the thermal reactions of bathoiodopsin were studied (Figure 1d). The photo-steady-state mixture containing mainly bathoiodopsin (curve 10) was warmed in the dark in a stepwise manner. As soon as the sample was warmed to a required temperature, it was recooled to −185 °C for measurement of the spectrum. The absorption spectrum began to change at −160 °C (curve 13) and gradually blue-shifted on further warming (curves 14–17). To clarify what reaction proceeded, a difference spectrum calculated by subtraction of curve 17 from curve 10 (curve 2′ in inset of Figure 1d) was compared with that between bathoiodopsin and iodopsin (curve 1′ in inset of Figure 1d) which was calculated by difference before and after a short irradiation (5 s) of iodopsin with the green light at −185 °C (curves 2 and 3 in Figure 1b, respectively). They were almost identical with each other, indicating that the spectral change induced by warming (Figure 1d) was mainly due to the reversion of bathoiodopsin to iodopsin. Then the sample was warmed to 0 °C (curve 18 in Figure 1a), followed by complete bleaching (curve 19). The loss of the absorbance at 545 nm (the isosbestic point between iodopsin and isoiodopsin at 0 °C) of the photo-steady-state mixture (curve 10) on warming was only 7.6% of that of the original iodopsin. This value was very close to a value (6.3%) measured in the digitonin extract (Yoshizawa, unpublished data). All these results obtained in the CHAPS–PC preparation were in essentially good agreement with those previously reported in the digitonin preparation (Yoshizawa & Wald, 1967).

N-Iodopsin. The *N*-iodopsin sample was subjected to an analysis similar to that of the iodopsin sample. The absorption spectrum of the *N*-iodopsin preparation was measured at 0 °C (curve 1 in Figure 2a). The absorption maximum of *N*-iodopsin at 0 °C was estimated to be 540 nm from the difference spectrum before and after complete bleaching shown in the inset of Figure 2a (curves 1 and 22 in Figure 2a).

Photochemical reactions of *N*-iodopsin at −185 °C are shown in Figure 2b,c. On irradiation of *N*-iodopsin with a green light (501 nm) at −185 °C, the gradual bathochromic shift of the spectrum proceeded (Figure 2b). A prolonged irradiation produced a photo-steady-state mixture containing mainly its batho intermediate (curve 11) as was observed by the irradiation of iodopsin (Figure 1b).

A similar photoreversibility among the original pigment, its batho intermediate, and its isopigment at −185 °C was observed in the case of *N*-iodopsin (Figure 2c). Namely, the irradiation of *N*-iodopsin (curve 2) with the green light induced a bathochromic spectral change due to formation of *N*-bathoiodopsin, resulting in the photo-steady-state mixture (curve 11). This mixture could be converted to a hypsochromic photoproduct, presumably *N*-isoiodopsin, by irradiation with a red light (>590 nm, curve 12). The subsequent irradiation with the green light induced a spectral change of the *N*-isoiodopsin to the photo-steady-state mixture (curve 13) whose spectrum coincided with curve 11.

Next, the photo-steady-state mixture containing mainly *N*-bathoiodopsin (curve 13 in Figure 1c) was warmed in a stepwise manner (curves 14–20 in Figure 2d); the spectrum began to shift to shorter wavelengths above −160 °C (curve

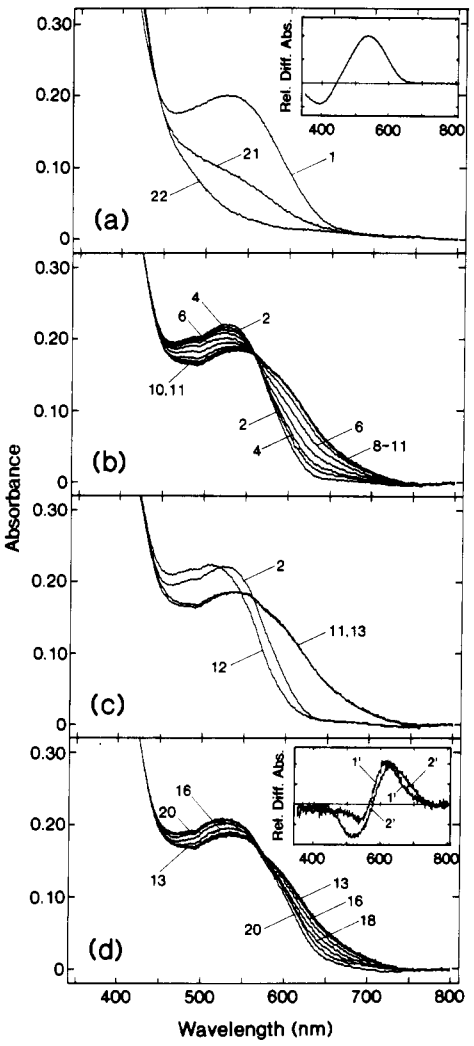

FIGURE 2: Photochemical and subsequent thermal reactions of *N*-iodopsin. (a) Absorption spectra of *N*-iodopsin and its bleaching products at 0 °C. The spectrum of the *N*-iodopsin–53% glycerol mixture was measured at 0 °C (curve 1) and then cooled to −185 °C [curve 2 in (b)], followed by a series of several irradiations with a different color light shown in (b) and (c). Then the photo-steady-state mixture containing mainly *N*-bathoiodopsin [curves 13 in (c) and (d)] was warmed to 0 °C after stepwise warming to −120 °C [curve 20 in (d)], followed by spectral measurement [curve 21 in (a)]. After extensive irradiation with red light (>590 nm) at 0 °C, the spectrum was measured (curve 22). Inset: Difference spectrum of *N*-iodopsin before and after complete bleaching with red light (curves 1 and 22, λ_{max} = 540 nm). (b) Formation of a photo-steady-state mixture containing mainly *N*-bathoiodopsin at −185 °C. The *N*-iodopsin preparation was cooled to −185 °C (curve 2) and then irradiated with green light (501 nm) for a total of 5, 10, 20, 40, 80, 160, 320, 640, and 1280 s (curves 3–11, respectively) until a photo-steady-state mixture was produced. (c) Photoreversibility among *N*-iodopsin, *N*-bathoiodopsin, and *N*-isoiodopsin at −185 °C. *N*-Iodopsin (curve 2) was irradiated with green light for 1280 s until the photo-steady-state mixture was produced (curve 11). Then the sample was irradiated with red light (>590 nm) for 3840 s (curve 12) for formation of mainly *N*-isoiodopsin, followed by reirradiation with green light for 1280 s (curve 13). (d) Conversion process of *N*-bathoiodopsin by stepwise warming. The photo-steady-state mixture containing mainly *N*-bathoiodopsin (curve 13) was warmed in the dark in a stepwise manner to −180, −170, −160, −150, −140, −130, and −120 °C (curves 14–20, respectively). All the spectra were measured at −185 °C after being warmed to the required temperature. Inset: Difference spectra between *N*-iodopsin and *N*-bathoiodopsin [curve 1′, which was calculated by subtracting curve 2 from curve 4 in (b)] and between curves 13 and 20 in (d) (curve 2′) which were normalized at their peaks. The two spectra were in disagreement with each other. Thus, the change of curve 13 to curve 20 shows the decay of *N*-iodopsin to putative *N*-lumiiodopsin.

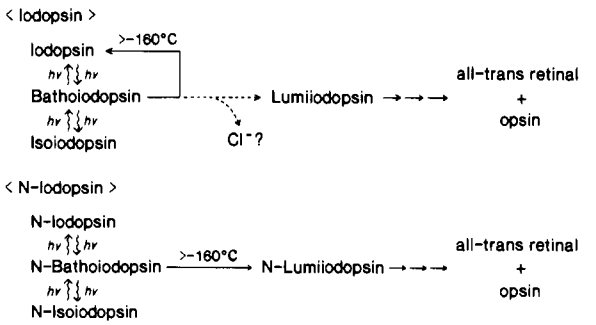

FIGURE 3: Photochemical and subsequent thermal reactions of iodopsin and *N*-iodopsin. Wavy lines, photochemical reactions. Straight lines, thermal reactions observed in the present low-temperature spectrophotometry. Dotted line, reactions which were hardly observed at low temperature. For details, see the text.

16). It should be noted that the difference absorption spectrum calculated by subtracting curve 20 from curve 13 (curve 2′ in the inset of Figure 2d) was different from that between *N*-bathoiodopsin and *N*-iodopsin (curve 1′ in Figure 2d) which was calculated from the difference before and after 10-s irradiation of *N*-iodopsin with the green light (curves 2 and 4 in Figure 2b, respectively). This fact clearly showed that the thermal product was not *N*-iodopsin. The absorption maximum of the product was located at a longer wavelength than that of the original *N*-iodopsin. On further warming to room temperature, the product in the mixture was bleached to *all-trans*-retinal and opsin, while residual *N*-iodopsin and *N*-isoiodopsin in the mixture still remained intact (curve 21 in Figure 2a). Therefore, *N*-bathoiodopsin should have been thermally bleached through at least one intermediate, presumably *N*-lumiiodopsin.

Discussion

In the present experiments at low temperature, bathoiodopsin produced by irradiation of iodopsin in the CHAPS–PC system exhibits the same thermal behavior as that in digitonin. Since the absorption maximum of iodopsin in CHAPS–PC was very closed to that in the membrane, it is expected that iodopsin in CHAPS–PC has a very similar structure to that in the membrane. This suggests that the reversion of the batho intermediate of iodopsin is the intrinsic property of the iodopsin molecule. *N*-Iodopsin is similar in photochemical behavior at −185 °C to the iodopsin system. Therefore, like iodopsin, the 11-cis chromophore of *N*-iodopsin would be photoisomerized to the all-trans form, resulting in the formation of *N*-bathoiodopsin which would be photoconverted to *N*-isoiodopsin having the 9-cis form as its chromophore. The Cl^- bound to iodopsin would have little effect on the photoreaction of iodopsin at −185 °C. However, in the presence of Cl^-, almost all the bathoiodopsin molecules produced at low temperature were reverted to the original iodopsin, while all the *N*-bathoiodopsin molecules were thermally converted to the next intermediate. The difference in reversion of the batho intermediate between both samples was not due to the ionic strength and glycerol concentration in the sample, because we confirmed that the iodopsin sample containing 630 mM NaCl and 53% glycerol showed the same results as those containing 140 mM NaCl and 71% glycerol (data not shown). The photochemical and thermal reactions of iodopsin and *N*-iodopsin are summarized in Figure 3.

Judging from the knowledge about rhodopsin, the conformational change of the protein moiety of rhodopsin begins with movement of the β-ionon ring of the chromophore in the conversion process of the batho to the lumi intermediate (Yoshizawa et al., 1987). If it were applicable to iodopsin,

the binding of Cl^- to the protein moiety of iodopsin may inhibit the conformational changes of the protein moiety near the β-ionon ring after photon absorption at low temperature, resulting in thermal isomerization of the all-trans chromophore of bathoiodopsin to the 11-cis form which is the chromophore of the original iodopsin.

It has been suggested that the chromophore of iodopsin should be located near the surface of the protein moiety compared to that of rhodopsin (Matsumoto et al., 1975). This idea was based upon the observations that the chromophore of iodopsin was more accessible to the chemical reagent than that of rhodopsin and that the regeneration rate of iodopsin was faster than that of rhodopsin (Matsumoto et al., 1975; Wald et al., 1955). The fact that the binding of Cl^-(s) to iodopsin caused about a 50-nm red-shift of the absorption spectrum (Knowles, 1976; Fager & Fager, 1979) indicates that the Cl^- binding site would be located near the chromophore. Although an indirect effect on the protein conformation which might be caused by Cl^- binding would be possible to explain partly this red-shift, the Cl^- itself is likely to have some direct effect on the chromophore. If so, it is inferred that the Cl^- would be released from the protein moiety in the early photobleaching process of iodopsin. Under this inference, the inhibition of conversion of bathoiodopsin to lumiiodopsin at low temperature is likely to be due to a blocking of release of Cl^- from the protein moiety by freezing of the surrounding water or by inhibition of the conformational change of the protein moiety near the Cl^- binding site (Figure 3).

When the Cl^- bound to iodopsin was replaced by NO_3^-, the absorption spectrum was blue-shifted. This spectral change was comparable to that when Cl^- was removed from the iodopsin preparation by extensive dialysis. Therefore, the removal of Cl^- or the replacement of Cl^- by NO_3^- causes a change in the electrostatic and/or steric interaction(s) between the chromophore and its nearby protein. The binding of NO_3^- to the protein moiety of iodopsin is likely to be weaker than that of Cl^-, because a large excess of NO_3^- is necessary for replacement of Cl^-. This suggests that NO_3^- may be easily released from the protein moiety in the photobleaching process. Thus, the thermal decay of *N*-bathoiodopsin to *N*-lumiiodopsin would result in release of NO_3^- from the protein. Though we have no direct evidence about the release of NO_3^-, this thermal decay would be alternatively explained by changing the shape of the chromophore binding site due to the binding of NO_3^- instead of Cl^-. Future low-temperature experiments on *N*-iodopsin and completely deionized iodopsin may provide additional evidence for release of the ion from the protein moiety of iodopsin in the early photobleaching process.

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Registry No. Cl^-, 16887-00-6; NO_3^-, 14797-55-8.

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