Effects of Chloride on Chicken Iodopsin and the Chromophore Transfer Reactions from Iodopsin to Scotopsin and B-Photopsin†

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ABSTRACT: Spectroscopic properties of chicken iodopsin were investigated in correlation with the concentration of chloride in digitonin extracts. When chloride in the extract was depleted by extensive dialysis, chloride-depleted iodopsin (absorption maximum, 512 nm) was formed. It was converted to chloride-bound iodopsin (absorption maximum, 562 nm) by the addition of chloride in the extract. There existed an equilibrium between two forms of iodopsin with a dissociation constant of 0.8 mM chloride. The chromophore-transfer reaction from iodopsin to scotopsin or B-photopsin, the protein moiety of chicken rhodopsin or chicken blue-sensitive cone pigment, respectively, in digitonin extract was also investigated in correlation with the concentrations of chloride, other monovalent and divalent anions, and detergent. The chromophore of chloride-depleted iodopsin was easily transferred to scotopsin in the extract, resulting in formation of rhodopsin. On the other hand, chloride-bound iodopsin was fairly stable even in the presence of scotopsin, indicating that the reaction is inhibited by binding of chloride to iodopsin. The chromophore-transfer reaction to B-photopsin was also observed from chloride-depleted iodopsin but not from chloride-bound iodopsin. The reaction was observable in the 10% digitonin extract as well as in the 2% digitonin extract. The reaction was also observed when 25 mM Na_2SO_4 was present in the mixture instead of NaCl, but was not when 67 mM $NaNO_3$ was present. All these facts suggest that the chloride binding site of iodopsin does not accept a divalent anion such as SO_4^{2+}, but does accept a monovalent anion such as Cl^- or NO_3^-, which causes inhibition of the chromophore transfer.

Iodopsin, one of the photopic visual pigments contained in cone cells of chicken retina, has several unique properties different from those of rhodopsin, the scotopic visual pigment in rod cell. Like rhodopsin, iodopsin has 11-*cis*-retinal as its chromophore. The Schiff base linkage in iodopsin between the chromophore and the putative lysine residue is, however, quite different in its accessibility to reagents of low molecular weight such as hydroxylamine, alum (Wald et al., 1955), and sodium borohydride (Matsumoto et al., 1975; Fager et al., 1975) from that in rhodopsin. The addition of 9-*cis*-retinal to an iodopsin extract replaced the 11-cis chromophore of iodopsin, resulting in the formation of 9-*cis*-iodopsin [chromophore-exchange reaction, Matsumoto et al. (1975)]. These facts, especially the occurrence of the chromophore-exchange reaction, suggested that the Schiff base linkage in iodopsin would be partly dissociated into 11-*cis*-retinal and R-photopsin in the chromophore binding site.

Another unique property of the chromophore–protein interaction in iodopsin was reported (Matsumoto et al., 1975) in which the 11-cis chromophore of iodopsin transferred to scotopsin (the protein moiety of rhodopsin) to form rhodopsin (chromophore-transfer reaction), if scotopsin was present in the iodopsin extract. Since the chromophore-transfer reaction would proceed by trapping of 11-*cis*-retinal by the large molecule, scotopsin, there would exist free 11-*cis*-retinal released from the chromophore binding site of iodopsin in the iodopsin–scotopsin extract. The chromophore-transfer reaction is a convenient way to trap small quantities of free 11-*cis*-retinal in the extract and it therefore measures the rate and extent of chromophore dissociation from iodopsin.

Meanwhile, it was reported that chloride affects the absorption spectrum of iodopsin and its photochemical behavior at low temperature. Iodopsin in digitonin extracts has an absorption maximum at 562 nm in the presence of chloride (chloride-bound iodopsin). When chloride was depleted from the extract, its absorption maximum was blue-shifted more than 40 nm [chloride-depleted iodopsin; Fager and Fager (1979), Knowles (1976), and Kato et al. (1984)]. Bathoiodopsin produced from chloride-bound iodopsin by irradiation at liquid nitrogen temperature thermally reverted to iodopsin (Yoshizawa & Wald, 1967), while that produced from iodopsin whose chloride binding site was occupied by NO_3^- was thermally converted to a next intermediate, presumably lumiiodopsin (Imamoto et al., 1989). These facts suggested that the chloride bound to iodopsin would play a crucial role in inducing an unique electrostatic and/or steric interaction between the chromophore and nearby protein in iodopsin as well as in its photochemical intermediates. Therefore, it is of interest to examine how the chromophore-transfer reaction is correlated to the presence of chloride in the binding site of iodopsin. The present work revealed that the dissociation constant of chloride in iodopsin was 0.8 mM and the chromophore-transfer reaction was dependent on the concentration of chloride in the extract.

Materials and Methods

Preparation of Various Kinds of Mixtures Containing Chloride-Depleted R-Photopsin, B-Photopsin, and/or Scotopsin. Various kinds of mixtures containing the protein moieties of iodopsin (R-photopsin), chicken blue-sensitive cone pigment (B-photopsin), and/or rhodopsin (scotopsin) were prepared by methods similar to those previously reported (Shichida et al., 1989). Chicken heads were purchased from a local poulterer within several hours after slaughter and

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brought to the laboratory in a light-tight icebox. All the procedures described below were done under normal room light and ice-chilled conditions. Usually 400 retinas were collected from the hemisected eyes and the photreceptor outer segments were isolated from the retinas by a sucrose flotation method [43% (w/v) sucrose in 10 mM HEPES[1] buffer, pH 6.6]. Since the outer segments thus obtained were contaminated by the oil droplets, they were washed several times with 10 mM HEPES buffer (pH 6.6) supplemented with 67 mM NaCl, followed by washing several times with petroleum ether after lyophilization. Then the opsins in the outer segments were extracted with 2% digitonin in HEPES buffer (pH 6.6).

The extract was further purified by Concanavalin A– (Con A–) Sepharose 4B affinity column chromatography as follows: The extract was loaded on a Con A affinity column (1 cm × 8 cm) equilibrated with 10 mM HEPES buffer supplemented with 67 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.1% digitonin. After the column was washed with ~100 mL of the equilibration buffer, opsin fractions were eluted from the column by the equilibration buffer supplemented with 5 mM α-methyl mannoside. The early fractions contained mainly R-photopsin with relatively small amounts of B-photopsin and scotopsin, while the late fractions contained R-photopsin and scotopsin. The fractions containing only scotopsin were eluted from the Con A–Sepharose 4B affinity column with the buffer supplemented with 100 mM α-methyl mannoside after complete elution of R-photopsin with the buffer supplemented with 5 mM α-methyl mannoside.

In order to prepare a mixture containing only R-photopsin and B-photopsin, the early fractions eluted with 5 mM α-methyl mannoside from the Con A column described above were concentrated by use of a ultrafiltration membrane (Amicon, YM-30), followed by dialysis against 1 L of 1 mM sodium phosphate buffer containing 0.1% digitonin (pH 6.6) overnight with several exchanges of fresh buffer. The dialysate was loaded on a DEAE-Sepharose column (1 cm × 16 cm) and eluted at a flow rate of 10 mL/h with the buffer used for the dialysis. Since only the scotopsin in the dialysate was adsorbed to the column under our experimental conditions, the eluates thus obtained contained only R-photopsin and B-photopsin.

For spectroscopic measurements, the eluates were concentrated by the use of ultrafiltration membranes and dialyzed against 1 L of 10 mM of HEPES buffer (pH 6.6) supplemented with 0.1% digitonin with three exchanges of fresh buffer in order to deplete the chloride and the α-methyl mannoside or to replace the phosphate by HEPES.

The amounts of R-photopsin, scotopsin, and B-photopsin in the samples thus obtained were estimated by partial bleaching of the samples in the presence of 10 mM hydroxylamine (H_2SO_4 form, and the pH was adjusted to 6.6 with NaOH) at 4 °C after the regeneration of opsins by the addition of a sufficient amount of 11-*cis*-retinal in the mixture (Schichida et al., 1989). The light source used for bleaching of iodopsin, rhodopsin, or chicken blue-sensitive cone pigment was a tungsten–halogen lamp (1 kW, Sanko); light was passed through either Toshiba glass cut-off filter VR-68, VO-59, or VO-52, before reaching the sample. A glass optical cell filled with water (light path, 6 cm) was placed as a heat shield between the filter and the light source.

Spectrophotometry. Absorption spectra of pigment samples were recorded on a Hitachi-330 spectrophotometer interfaced with an NEC PC-9801F computer and CD spectra of the samples on a Jasco J-20 spectropolarimeter.

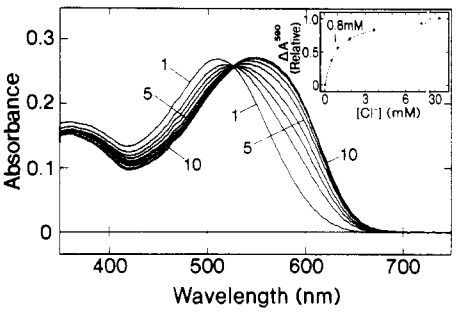

FIGURE 1: Conversion of chloride-depleted iodopsin to its bound form by successive addition of NaCl to the extract. A slight excess of 11-*cis*-retinal was added to the R-photopsin–scotopsin mixture (3:1) to generate chloride-depleted iodopsin and rhodopsin (curve 1). Then a small amount of 3 M NaCl solution was successively added to the mixture (curves 2–10). The concentrations of NaCl in the mixture were 0, 0.49, 0.95, 1.9, 3.7, 7.2, 16, 36, 75, and 120 mM (curves 1–10, respectively). The dilution effect due to the addition of 3 M NaCl was corrected in each spectrum. There is a clear isosbestic point at 528 nm, indicating that these spectral changes are due to the conversion of only chloride-depleted iodopsin to the chloride-bound form. Inset: Increases in relative absorbance at 590 nm were plotted against concentrations of NaCl in the mixture. An arrow indicates the NaCl concentration at which the amount of chloride-depleted iodopsin is equal to that of chloride-bound iodopsin.

RESULTS

Conversion of Chloride-Depleted Iodopsin to Chloride-Bound Iodopsin. Figure 1 shows the spectral change of iodopsin dependent on the concentration of chloride in the medium. To ensure that the pigments are in fully regenerated state, a small excess of 11-*cis*-retinal was added to the preparation containing the chloride-depleted R-photopsin and scotopsin (3:1[2]), followed by incubation for 5 h at 20 °C (curve 1). The successive addition of 3 M NaCl solution in 10 mM HEPES buffer (pH 6.6) to the preparation caused a gradual spectral shift to the red. It was confirmed from a separate experiment on a purified scotopsin preparation that no spectral change was observed in rhodopsin by the addition of 3 M NaCl. Thus the spectral changes shown in Figure 1 should be due to the conversion of chloride-depleted iodopsin to chloride-bound iodopsin.

A relationship between the increase of absorbance at 590 nm and the concentration of chloride in the medium is shown in the inset of Figure 1. If iodopsin has a single chloride binding site for the spectral change, an equilibrium between chloride-depleted iodopsin (Iod) and choride-bound iodopsin (Iod-Cl) can be expressed as follows:

$$\text{Iod} + \text{Cl}^- \rightleftarrows \text{Iod-Cl}^-$$

$$K = [\text{Iod}][\text{Cl}^-]/[\text{Iod-Cl}^-]$$

in which K is the dissociation constant. The increase of absorbance at 590 nm (ΔA^{590}) by the addition of NaCl is related to the amounts of chloride-depleted and -bound iodopsins in the preparation as expressed by the following equations:

$$\Delta A^{590} = (\epsilon_b{}^{590} - \epsilon_d{}^{590})[\text{Iod-Cl}^-]$$

$$\Delta A_0{}^{590} = (\epsilon_b{}^{590} - \epsilon_d{}^{590})I_t$$

in which $\epsilon_b{}^{590}$ and $\epsilon_d{}^{590}$ are the extinction coefficients of chloride-bound and -depleted iodopsins at 590 nm, $\Delta A_0{}^{590}$ is

[1] Abbreviation: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] The ratio between R-photopsin and scotopsin was estimated to be 3:1 from maximal absorbances of iodopsin and rhodopsin after the complete regeneration.

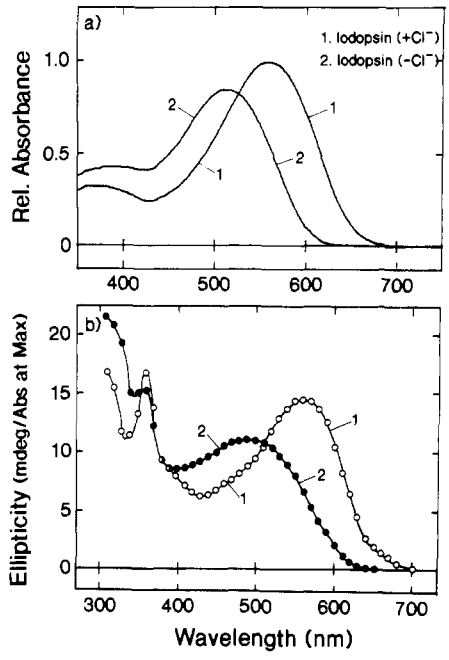

FIGURE 2: Absorption and CD spectra of chloride-bound iodopsin (1) and the chloride-depleted form (2). The absorption and CD spectra of chloride-depleted iodopsin are shown relative to those of chloride-bound iodopsin. The absorption maxima of the pigments are located at 562 (chloride-bound iodopsin) and 512 nm (chloride-depleted iodopsin).

the absorbance difference when all chloride-depleted iodopsin was converted to the chloride-bound form, and I_t is the total amount of iodopsin in the mixture. From these equations, the following equation can be derived:

$$\frac{1}{A^{590}} = \frac{1}{A_0^{590}}\left(1 + \frac{K}{[Cl^-]}\right)$$

Therefore, the reciprocal of absorbance increase is proportional to the reciprocal of chloride concentration in the mixture. In fact, a linear relation was obtained, indicating that iodopsin has a single chloride binding site. The dissociation constant of chloride in iodopsin was estimated to be 0.8 mM (Figure 1, inset).

Absorption and CD Spectra of Iodopsins. The absorption and CD spectra of chloride-bound iodopsin and its chloride-depleted form were shown in Figure 2. The absorption spectrum of chloride-bound iodopsin was obtained by the addition of a small amount of 11-*cis*-retinal to the R-photopsin–scotopsin mixture in the presence of 67 mM NaCl. Under these conditions, only chloride-bound iodopsin was regenerated, because the regeneration rate of chloride-bound iodopsin was much faster than that of rhodopsin (Wald et al., 1955). On the other hand, the absorption spectrum of chloride-depleted iodopsin could not be obtained by the application of a similar procedure to the R-photopsin–scotopsin mixture in the absence of NaCl, because chloride-depleted iodopsin, which had been rapidly regenerated by the addition of 11-*cis*-retinal, was readily bleached by the transfer reaction of the 11-cis chromophore to scotopsin in the mixture (see below). The absorption spectrum of chloride-depleted iodopsin was therefore calculated from the absorption spectrum of chloride-bound iodopsin and the difference spectrum between chloride-depleted iodopsin and chloride-bound iodopsin obtained by subtraction of curve 1 from curve 10 in Figure 1. The calculation was made on the assumption that chloride-depleted iodopsin has no absorbance at wavelengths longer than 650 nm.

The absorption spectrum of chloride-depleted iodopsin was also estimated by application of the partial bleaching method to a mixture containing only chloride-depleted iodopsin and chicken blue-sensitive cone pigment without any rhodopsin. Since chicken blue-sensitive cone pigment has an absorption maximum (450 nm) at a shorter wavelength than that of chloride-depleted iodopsin and does not absorb light at wavelengths longer than 580 nm, the absorption spectrum of chloride-depleted iodopsin (difference spectrum between chloride-depleted iodopsin and its bleaching product) could be obtained by irradiation of the mixture with light at a wavelength longer than 580 nm. The spectrum obtained was in good agreement in the spectral region above 440 nm with that calculated from the spectrum of chloride-bound iodopsin and the difference spectrum upon conversion from chloride-depleted iodopsin to its chloride-bound form.

CD spectra of the mixture of chloride-depleted iodopsin and rhodopsin and that of chloride-bound iodopsin and rhodopsin were measured with aliquots of the same sample as used for measurment of the absorption spectra shown in Figure 1 (curves 1 and 10, respectively). Then the CD spectra of chloride-depleted and -bound iodopsins were calculated by subtracting the CD spectra of rhodopsin present in the mixtures from those of the mixtures. The CD spectrum of rhodopsin was separately measured on a purified rhodopsin sample (data not shown).

Chromophore-Transfer Reaction from Chloride-Depleted Iodopsin to Scotopsin or B-Photopsin. The chromophore-transfer reaction from iodopsin to scotopsin in samples containing various concentrations of chloride was investigated (Figure 3). An R-photopsin–scotopsin mixture (1:2) was first dialyzed against the chloride-free buffer to prepare a chloride-depleted R-photopsin–scotopsin mixture. A sufficient amount of 11-*cis*-retinal for binding of almost all the R-photopsin in the mixture was added to the mixture and absorption spectra were repeatedly recorded at intervals of 8 min (curves 1–7 in Figure 3a). The spectrum gradually shifted to the blue with a large increase around 500 nm and a small decrease around 580 nm. Finally, hydroxylamine (a final concentration of 100 mM) was added to the sample. This caused ~15% decrease of absorbance at 512 nm owing to the decomposition of the chloride-depleted iodopsin. The residual pigment in the sample was identified as rhodopsin regenerated during the repeat scanning. When the hydroxylamine was added to the sample immediately after the addition of 11-*cis*-retinal, the absorbance at 512 nm of the original sample decreased more than 90%. These facts indicated that the chromophore of chloride-depleted iodopsin was transferred to scotopsin during the repeat scanning.

In order to test the chloride dependency of the chromophore-transfer reaction, R-photopsin–scotopsin mixtures (1:3) with various chloride concentrations were prepared.

The absorption maximum of the sample containing 1 mM NaCl (curve 1 in Figure 3b) measured immediately after the addition of 11-*cis*-retinal lay at ~530 nm owing to the formation of an equilibrium mixture of chloride-depleted and -bound forms of iodopsin. Then the maximum shifted to the blue. The presence of a clear isosbestic point at 535 nm indicates occurrence of the chromophore-transfer reaction from the mixture of chloride-depleted iodopsin and its bound form to scotopsin in the sample and the rate of the shift from chloride-bound iodopsin to its depleted form is much faster than that of the chromophore-transfer reaction.

A similar spectral change was observed in the sample containing 6 mM NaCl (Figure 3c), but the amount of rhodopsin

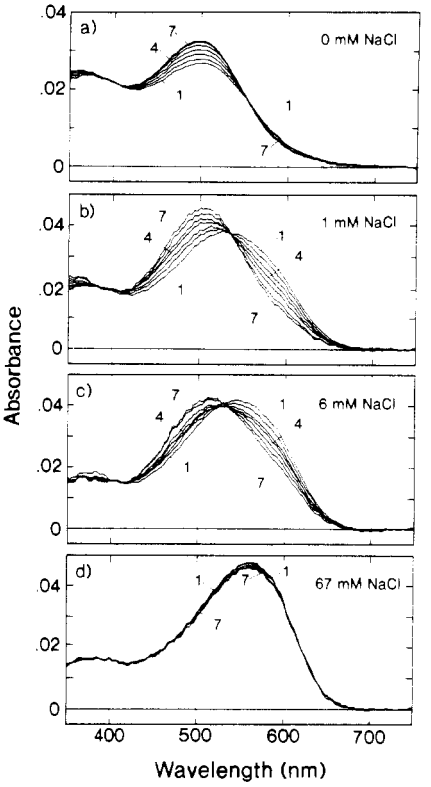

FIGURE 3: Relationship between chromophore-transfer reaction and concentration of NaCl in the sample. (a) Immediately after the addition of a small amount of 11-*cis*-retinal to a chloride-depleted R-photopsin–scotopsin mixture (1:2), the absorption spectrum was measured (curve 1) and then recorded at time intervals of 8 min (curves 2–7). (b–d) A chloride-depleted R-photopsin–scotopsin mixture (1:3) was divided into three fractions, to each of which was added 1 (b), 6 (c), or 67 mM (d) NaCl in 10 mM HEPES buffer. Immediately after the addition of a small amount of 11-*cis*-retinal to each fraction, the absorption spectrum was measured (curve 1) and then recorded successively at intervals of 8 min (curves 2–7). Details are given in the text.

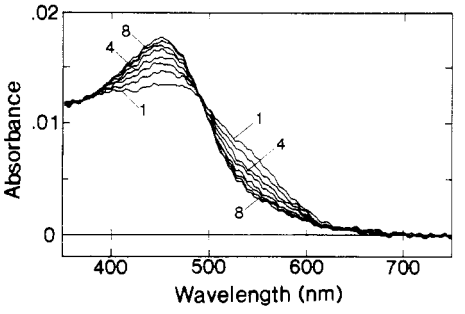

FIGURE 4: Chromophore-transfer reaction from chloride-depleted iodopsin to B-photopsin. A small amount of 11-*cis*-retinal was added to a chloride-depleted R-photopsin–B-photopsin mixture, followed by measurement of the spectra repeatedly at intervals of 8 min (curves 1–8). There is an isosbestic point at 486 nm.

regenerated in 6 mM NaCl during the incubation was less than that in 1 mM NaCl. An isosbestic point was present at 531 nm. The difference in wavelength of isosbestic point between the samples containing 1 and 6 mM NaCl would be explained by the difference in composition between chloride-bound and -depleted forms. Finally, the samples containing 67 mM NaCl (Figure 3d) as well as 100 mM NaCl (data not shown) did not display the chromophore-transfer reaction.

The chromophore-transfer reaction from chloride-depleted iodopsin to B-photopsin was also observed (Figure 4). It should be noted that an isosbestic point lay at 486 nm, a much shorter wavelength than those observed in the iodopsin–scotopsin mixture.

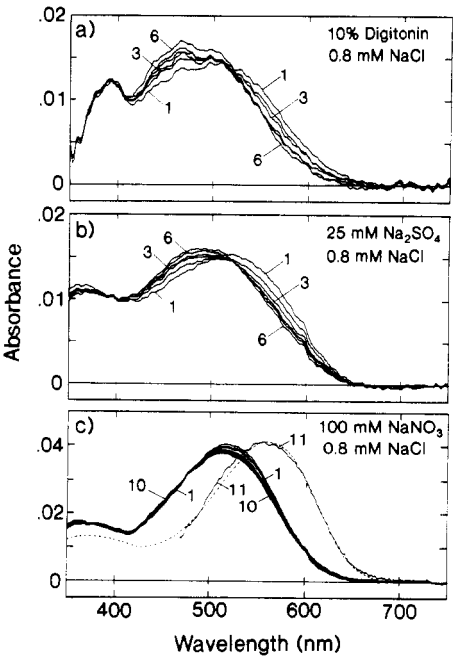

FIGURE 5: Effects of digitonin, divalent anion, and monovalent anion on the chromophore-transfer reaction. The mixture composed of chloride-depleted R-photopsin, scotopsin, and B-photopsin was mixed with 10 mM HEPES buffer supplemented with 3 M NaCl to give a final concentration of 0.8 mM NaCl. (a) The digitonin concentration in the mixture was raised by the addition to the mixture of the digitonin powder, which was prepared by lyophilizing a supernatant of 2.5% digitonin solution obtained by centrifugation (116000*g* for 30 min) (Bridges, 1977). Then absorption spectra of the sample were repeatedly measured at intervals of 8 min (curves 1–6) immediately after the addition of 11-*cis*-retinal. (b) The mixture was mixed with 10 mM HEPES buffer supplemented with 1 M Na_2SO_4 to give a final concentration of 25 mM Na_2SO_4. Immediately after the addition of 11-*cis*-retinal to the sample, the absorption spectra were repeatedly measured at intervals of 8 min (curves 1–6). (c) The mixture was mixed with 10 mM HEPES buffer supplemented with 3 M $NaNO_3$ to give a final concentration of 100 mM $NaNO_3$. Immediately after the addition of 11-*cis*-retinal to the sample, the absorption spectra were repeatedly measured at intervals of 8 min (curves 1–10). After the final spectrum was measured (curve 10), 3 M NaCl in 10 mM HEPES buffer was added to the sample to give a final concentration of 100 mM NaCl in order to confirm the presence of iodopsin (curve 11). The absorption spectrum of chloride-bound iodopsin (same as curve 1 in Figure 2a) is shown as the dotted line.

These results showed that the chromophore-transfer reaction easily occurred when the concentration of NaCl in the sample was decreased. In order to check other factors that promote the chromophore-transfer reaction, the following three experiments (Figure 5) were performed in the presence of 0.8 mM NaCl, where iodopsin is in an equilibrium between chloride-depleted and -bound forms.

First, the possibility that the chromophore-transfer reaction might be blocked when the digitonin concentration in the sample is high (Matsumoto et al., 1975) was examined by increasing digitonin concentration in the sample up to 10% (Figure 5a). As shown in Figure 5a, the chromophore-transfer reaction from chloride-depleted iodopsin to scotopsin or B-photopsin was observed, indicating that digitonin does not affect the reaction.

Second, the effect of ionic strength of the sample on the chromophore-transfer reaction was investigated by the addition of 25 mM Na_2SO_4 to the sample followed by successive measurements of the spectra after the addition of 11-*cis*-retinal. As shown in Figure 5b, the chromophore-transfer reaction was observed even in the presence of 25 mM sulfate. Then 100 mM NaCl was added to the sample. No shift of the absorption spectrum was observed. These facts clearly demonstrated that

the chromophore-transfer reaction is independent of the ionic strength of the sample.

Third, we examined the effect of another monovalent anion on the chromophore-transfer reaction (Figure 5c). When 100 mM $NaNO_3$ was added to the sample followed by the addition of 11-*cis*-retinal, the absorption maximum of the sample was located at 520 nm, and then the spectrum gradually shifted to the blue with some decrease of the peak absorbance during the dark incubation, possibly owing to the replacement of chloride in chloride-bound iodopsin by nitrate. The addition of 100 mM NaCl to the sample after the incubation shifted the absorption spectrum to the red (curve 11 in Figure 5c), owing to the formation of chloride-bound iodopsin. The fact that the absorption spectrum measured after the addition of NaCl (curve 11 in Figure 5c) is almost the same in shape to that of chloride-bound iodopsin clearly showed that chromophore transfer did not occur in the sample during the incubation. Thus, the addition of $NaNO_3$ to the sample as well as NaCl blocks the chromophore-transfer reaction.

Discussion

The present experiments clearly show that the chromophore-transfer reaction from iodopsin to scotopsin or B-photopsin occurs when the chloride-depleted form of iodopsin is present in the sample. The chromophore-transfer reaction did occur in the presence of 10% digitonin, refuting the early suggestion (Matsumoto et al., 1975) that the reaction would be inhibited by digitonin in the sample. The reaction also proceeded in the sample containing 25 mM Na_2SO_4, whose ionic strength is equivalent to that of the sample containing 75 mM NaCl, though it was inhibited in the sample containing 67 and 100 mM NaCl. Therefore, the detergent and the ionic strength of the medium do not affect the chromophore-transfer reaction.

As already described, the chromophore-transfer reaction would proceed by way of trapping of 11-*cis*-retinal by the large molecule, scotopsin. Since the 11-*cis*-retinylidene chromophore of rhodopsin is embedded in the center of the α-helix region of rhodopsin (Hargrave et al., 1984), it is unrealistic that 11-*cis*-retinal was captured by scotopsin through direct contact with iodopsin, even though the chromophore binding site was supposed to be present near the surface of iodopsin (Fukada et al., 1990). Therefore, in the sample of chloride-depleted iodopsin, some amount of 11-*cis*-retinal is probably present in free form[3] in the medium. The binding of chloride to the protein moiety of chloride-depleted iodopsin may induce a conformational change of the chromophore binding site such that release of 11-*cis*-retinal to the medium is prevented.

Chloride-depleted iodopsin has a smaller CD at the α-band than that of chloride-bound iodopsin, suggesting that the chromophore in chloride-depleted iodopsin is not as twisted as that in chloride-bound iodopsin. Thus, the steric interaction between the chromophore and protein in chloride-depleted iodopsin is smaller than that found in chloride-bound iodopsin.

A reaction scheme illustrating the chromophore-transfer reaction and the hydroxylamine accessibility of iodopsin is presented is Figure 6 in which the equilibrium between chloride-depleted iodopsin and its chloride-bound form and that between chloride-depleted iodopsin and 11-*cis*-retinal plus R-photopsin are shown.

[3] Since 11-*cis*-retinal is insoluble in the buffer solution without detergent, the phrase "free form" means that 11-*cis*-retinal does not bind in the chromophore binding site of iodopsin. It may exist in the detergent micelle or bind to some amino acid residues on the surface of the protein and lipid.

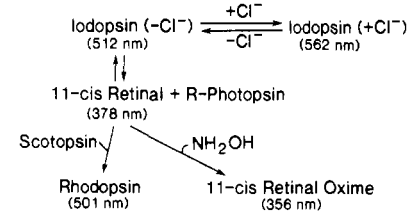

FIGURE 6: Reaction scheme of iodopsin in the presence of scotopsin or hydroxylamine.

Iodopsin is in the stable form (chloride-bound form) at a high concentration of chloride. When the concentration of chloride in the mixture is lowered, the equilibrium between chloride-bound and -depleted forms shifts to the latter, which is partly dissociated into 11-*cis*-retinal and R-photopsin. If scotopsin or hydroxylamine is present in the sample containing chloride-depleted iodopsin, the 11-*cis*-retinal would be trapped to form rhodopsin or retinal oxime, respectively.

When the absorption spectrum of the sample containing both 0.8 mM NaCl and 100 mM $NaNO_3$ was measured immediately after the addition of 11-*cis*-retinal, the absorption maximum was located at ~520 nm (Figure 5c, curve 1), whose value was similar to that of the sample containing only 0.8 mM NaCl. Then the spectrum gradually shifted to the shorter wavelengths concurrent with the decrease of absorbance during the incubation (Figure 5c, curves 1–10) and finally reached 512 nm, a value in agreement with that of chloride-depleted iodopsin. As already stated, this spectral change was not due to the chromophore-transfer reaction from iodopsin to scotopsin because the further addition of NaCl to the incubated sample resulted in the spectrum that was almost the same in shape as that of chloride-bound iodopsin (Figure 5c, curve 11). Thus, the spectral change during the incubation may be due to a slow exchange of chloride for nitrate or a slow conformational change without ion exchange. If the former is correct, the first appearance of the 520-nm form of iodopsin in the presence of nitrate, possibly as a mixture of chloride-bound and nitrate-bound forms, suggests that the binding constant of nitrate relative to chloride is smaller in R-photopsin than in iodopsin.

The chloride effect on the absorption spectrum was also observed in one of the Gecko visual pigments, although the shift of absorption maximum was not so large (Crescitelli, 1977). Recently the chromophore-exchange reaction was reported in this pigment (Crescitelli, 1989). Thus, this pigment is similar to iodopsin in the nature of the chromophore binding site and Schiff base linkage between chromophore and protein. There are, however, some differences in spectroscopic properties between them. The absorption maximum of the chloride-depleted form of Gecko pigment was further blue-shifted by the addition of $NaNO_3$ (Crescitelli, 1980), while that of chloride-depleted iodopsin was not.

The fact that monoclonal antibodies against iodopsin did not react with Gecko visual pigment (Schichida et al., 1989) also suggested that there are some differences in protein moiety between these pigments.

Halorhodopsin, one of the retinoid pigments in *Halobacterium halobium*, also has a chloride effect on the absorption spectrum (Ogurusu et al., 1982). It was suggested that halorhodopsin has two chloride binding sites in its protein moiety, one of which is highly specific to chloride and the other of which is relatively nonspecific to the monovalent anion (Schobert et al., 1986). It should be noted that the absorption spectrum of halorhodopsin is dependent on the binding of ions in both sites, while iodopsin has a single binding site that affects the absorption spectrum of iodopsin. Our future research will

determine whether or not R-photopsin has another anion binding site that does not affect the absorption spectrum.

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REFERENCES

Bridges, C. D. B. (1977) *Vision Res. 17*, 301-302.
Crescitelli, F. (1977) in *Handbook of Sensory Physilogy* (Crescitelli, F., Ed.) Vol. VII/5, pp 391-449, Springer-Verlag, Berlin.
Crescitelli, F. (1980) *Vision Res. 20*, 937-945.
Crescitelli, F., & Karvaly, B. (1989) *Exp. Eye Res. 49*, 43-48.
Fager, L. Y., & Fager, R. S. (1979) *Exp. Eye Res. 29*, 401-408.
Fager, R. S., Kandel, M., Heppner, T., & Abrahamson, E. W. (1975) *Vision Res. 15*, 741-742.
Fukada, Y., Okano, T., Shichida, Y., Yoshizawa, T., Trehan, A., Mead, D., Denny, M., Asato, A. E., & Liu, R. S. H. (1990) *Biochemistry 29*, 3133-3140.
Hargrave, P. A., McDowell, J. H., Feldman, R. J., Atkinson, P. H., Mohana, J. K., & Argos, P. (1984) *Vision Res. 24*, 1487-1499.
Hubbard, R., Brown, P. K., & Bownds, D. (1971) *Methods Enzymol. 18*, 615-653.
Imamoto, Y., Kandori, H., Okano, T., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1989) *Biochemistry 28*, 9412-9416.
Kato, T., Sasayama, S., Shichida, Y., & Yoshizawa, T. (1984) *Zool. Sci. 1*, 862.
Knowles, A. (1976) *Biochem. Biophys. Res. Commun. 73*, 56-62.
Matsumoto, H., Tokunaga, F., & Yoshizawa, T. (1975) *Biochim. Biophys. Acta 404*, 300-308.
Ogurusu, T., Maeda, A., Sasaki, N., & Yoshizawa, T. (1982) *Biochim. Biophys. Acta 682*, 446-451.
Schobert, B., Lanyi, J. K., & Oesterhelt, D. (1986) *J. Biol. Chem. 261*, 2690-2696.
Shichida, Y., Taniguchi, Y., Kuwata, O., Fukada, Y., Yoshizawa, T., Horiuchi, S., & Takeichi, M. (1989) *Exp. Eye Res. 48*, 281-293.
Wald, G., Brown, P. K., & Smith, P. H. (1955) *J. Gen. Physiol. 38*, 623-680.
Yoshizawa, T., & Wald, G. (1967) *Nature 214*, 566-571.

Identification of Cellular Proteins Binding to the Scrapie Prion Protein†

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ABSTRACT: The scrapie prion protein (PrP^{Sc}) is an abnormal isoform of the cellular protein PrP^{C}. PrP^{Sc} is found only in animals with scrapie or other prion diseases. The invariable association of PrP^{Sc} with infectivity suggests that PrP^{Sc} is a component of the infectious particle. In this study, we report the identification of two proteins from hamster brain of 45 and 110 kDa (denoted *Pr*P *li*gands Pli 45 and Pli 110) which were able to bind to PrP 27-30, the protease-resistant core of PrP^{Sc} on ligand blots. Pli 45 and Pli 110 also bound PrP^{C}. Both Pli's had isoelectric points of ~5. The dissociation rate constant of the Pli 45/PrP 27-30 complex was 3×10^{-6} s^{-1}. Amino acid and protein sequence analyses were performed on purified Pli 45. Both the composition and the sequence were almost identical with those predicted for mouse glial fibrillary acidic protein (GFAP). Furthermore, antibodies to Pli 45 reacted with recombinant GFAP. The identification of proteins which interact with the PrP isoforms in normal and diseased brain may provide new insights into the function of PrP^{C} and into the molecular mechanisms underlying prion diseases.

Infectious particles causing scrapie and other prion diseases are composed largely of the scrapie prion protein (PrP^{Sc}),[1] which is an abnormal isoform of a cellular protein (PrP^{C}) (Bolton et al., 1982; Prusiner et al., 1983; Oesch et al., 1985). Extended proteolysis destroys PrP^{Sc} (or PrP 27-30) and diminishes infectivity with similar kinetics (McKinley et al., 1983). Genetic analysis in mice has linked different alleles of the PrP gene to a gene controlling the incubation time (Carlson et al., 1986; Westaway et al., 1987). In humans, a point mutation that leads to an amino acid substitution in PrP has been linked to familial Gerstmann-Sträussler syndrome (Hsiao et al., 1989).

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[1] Abbreviations: PrP^{Sc}, scrapie isoform of the prion protein; PrP 27-30, protease-resistant core of PrP^{Sc}; PrP^{C}, cellular isoform of the prion protein; GFAP, glial fibrillary acidic protein; DLPC, detergent-lipid-protein complexes; Pli, PrP ligand.

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