Effect of Anion Binding on the Thermal Reverse Reaction of Bathoiodopsin: Anion Stabilizes Two Forms of Iodopsin†

Takahiro Hirano,‡,§ Hiroo Imai,‡,# and Yoshinori Shichida*,‡,#

Department of Biophysics, Graduate School of Science, Kyoto University, and Core Research for Evolutional Science and Technology, Japan Science and Technology, Kyoto 606-8502, Japan

Received April 28, 2003; Revised Manuscript Received August 16, 2003

ABSTRACT: The thermal reactions of the bathoproduct of the long wavelength sensitive visual pigment iodopsin were investigated under various anionic and environmental conditions, to get an insight into the mechanism leading to the unusual thermal isomerization of the retinal chromophore from the trans to the 11-cis form at very low temperatures (−160 °C). The all-trans chromophore of the bathoiodopsin produced from iodopsin in the presence of chloride thermally reverted to the 11-cis form, while in the presence of nitrate it kept its all-trans configuration upon warming. Different protein environments, either in a detergent or in phosphatidylcholine (PC) liposomes, did not change the reaction characteristics of the bathoiodopsins under the two anionic conditions. However, reaction characteristics of bathoiodopsins produced in the absence of small anions were dependent on the environment. The trans-to-cis isomerization occurred upon warming of bathoiodopsin in the presence of detergent but not in liposomes. Spectral measurements revealed that iodopsin in the absence of small anions is a mixture of two spectrally distinct forms that exhibit absorption maxima and reaction characteristics similar to those of chloride-bound and nitrate-bound iodopsins, respectively. Thus, iodopsin exhibits two conformational states, each of which is stabilized by the binding of chloride and nitrate, respectively.

Iodopsin (iod)[1] is a visual pigment present in chicken red-sensitive cone photoreceptor cells (*1*) and belongs to the group of long-wavelength-sensitive visual pigments (group L) among the four groups (S (Short), M1 (Middle1), M2 (Middle2), and L (Long)) of cone visual pigments (*2*−*4*). Because iodopsin is 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 rod and cone photoreceptor cells.

Like the rod visual pigment rhodopsin, iodopsin has an 11-*cis*-retinal as its chromophore and converts to the transducin-activating state (*5*, *6*) through cis−trans photoisomerization of the chromophore in the primary intermediates (*7*−*9*), followed by the protein conformational changes (*10*). However, there are distinct differences in molecular properties between these pigments (*1*, *11*), which originate from the difference in amino acid sequence (*2*, *4*). The most prominent difference is that unlike rhodopsin, iodopsin has a chloride-binding site in its protein moiety and removal of the chloride or its exchange with nitrate causes a blue-shift of the absorption spectrum (chloride effect) (*12*−*15*). The presence of this chloride-binding site is a general feature of cone visual pigments belonging to the L group (*16*) except in rodents (*17*−*21*).

Another difference is that the batho-intermediate of iodopsin (bathoiodopsin) produced at −196 °C thermally reverts to the original iodopsin upon warming, while that of rhodopsin bleaches to all-*trans*-retinal and opsin through several intermediates (*7*). Because the chromophore of bathoiodopsin has an all-trans configuration like that of bathorhodopsin (*7*, *9*, *22*−*24*), these results indicate that the thermal isomerization of the chromophore from the all-trans to 11-cis forms occurs at an extremely low temperature (−160 °C) in the iodopsin system. Therefore, it is of interest to investigate the mechanism leading to the thermal isomerization at such a low temperature.

It has been frequently indicated that there is some relationship between chloride binding and the thermal isomerization of the chromophore. When chloride in the binding site was replaced with nitrate, bathoiodopsin was converted to the next intermediate lumiiodopsin without thermal isomerization of the chromophore (*25*). These results suggest that the chloride binding affects the isomerization reaction in bathoiodopsin. However, subsequent investigation using a gluconate, a large anion that was expected not to

† This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

* To whom correspondence should be addressed. Phone: +81−75−753−4213. Fax: +81−75−753−4210. E-mail: shichida@photo2.biophys.kyoto-u.ac.jp.

‡ Department of Biophysics.

Core Research for Evolutional Science and Technology.

§ Present Address: Suntory Institute for Bioorganic Research, 1-1 Wakayamadai, Shimamoto, Osaka 618-8503, Japan.

[1] Abbreviations: iod, iodopsin; batho, bathoiodopsin; iod·Cl, chloride-bound iodopsin; iod·NO_3, nitrate-bound iodopsin; iod·na, iodopsin binding no anion; iod·glu, iodopsin in the presence of gluconate ion; LWC, the long-wavelength component; SWC, the short-wavelength component; FTIR, Fourier transform infrared; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; CHAPS/PC, buffer solution that contains a mixture of CHAPS and PC; Con A, concanavalin A; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; KIU, kallikrein inhibitor unit; HPLC, high-pressure liquid chromatography.

10.1021/bi034671w CCC: $25.00
Published on Web 10/07/2003

bind to the chloride-binding site of iodopsin, demonstrated that the anion-unbound bathoiodopsin also showed thermal isomerization upon warming (*26*). These results suggested that nitrate, a lyotropic anion, has some unusual effect on the thermal isomerization in iodopsin intermediates.

To investigate the difference in chromophore-opsin interaction between the chloride-bound and nitrate-bound bathoiodopsins that show thermal isomerization and no isomerization, respectively, we have previously measured FTIR spectra of these bathoiodopsins in PC liposomes (*23*, *24*). The results showed that only the chloride-bound bathoiodopsin exhibited the specific FTIR band showing the interaction between C_{14} position of the chromophore and nearby protein, while the chromophore structure including the all-trans configuration was similar in each other as evidenced by the similar positions of the FTIR marker bands except for the above band in the FTIR spectra (*23*). In addition, subsequent FTIR study indicated that the anion-unbound bathoiodopsin exhibited the FTIR spectrum almost the same as that of the nitrate-bound bathoiodopsin and did not show the band that reflects the specific interaction observed in the chloride-bound bathoiodopsin (*24*). Thus, the FTIR experiments revealed that the chloride-bound bathoiodopsin exhibits a chromophore−opsin interaction different from those in the nitrate-bound and anion-unbound bathoiodopsins, while the latter two show the similar chromophore−opsin interaction. These results appeared to indicate no relationship between the chromophore−opsin interaction in bathoiodopsin and reaction pathway the chromophore of bathoiodopsin takes, because like the chloride-bound bathoiodopsin, the anion-unbound bathoiodopsin was reported to show thermal isomerization (*26*).

However, during the course of FTIR experiments, we found that the anion-unbound iodopsin in PC liposome exhibited a visible absorption maximum considerably different from that in the CHAPS/PC system and almost the same as that of nitrate-bound iodopsin (*24*). Thus, it is possible that the chromophore−opsin interaction in bathoiodopsin is dependent on environmental conditions, which could cause the apparent discrepancy between the chromophore−opsin interaction in bathoiodopsin and the reaction pathway taken by the chromophore of bathoiodopsin.

In the present study, we have investigated in detail the effects of anions on the absorption spectrum of iodopsin and thermal isomerization of the chromophore upon the warming of bathoiodopsin. We also analyzed the chromophore compositions of the iodopsin samples under the various anionic and environmental conditions before and after irradiation at −196 °C. The results clearly show that only the chloride-bound bathoiodopsin exhibits thermal isomerization in both PC liposomes and the CHAPS/PC environment, while anion-unbound bathoiodopsin exhibits thermal isomerization in the CHAPS/PC environment but exhibits bleaching without isomerization of the chromophore in PC liposomes. Taken together with the results of FTIR measurements on bathoiodopsins reported recently (*24*), these results suggested that the chromophore−opsin interaction in bathoiodopsin, especially around the C_{14} position, correlated well with the occurrence of thermal isomerization. On the basis of these results and the effect of temperature on the spectral features of iodopsin samples, the mechanism of the thermal isomerization of the chromophore in bathoiodopsin is discussed.

MATERIALS AND METHODS

Sample Preparation. Iodopsin was extracted from about 2000 chicken retinas by a mixture of CHAPS and PC and purified by column chromatography (*27*). In the present study, the iodopsin samples purified on Con A-Sepharose affinity and SP−Sepharose ion exchange columns (Pharmacia) were further purified by repeated application to a Con A-Sepharose column. That is, each sample was applied to a Con A-Sepharose column equilibrated with buffer A [20% glycerol (w/v), 0.6% CHAPS, 0.8 mg/mL PC, 50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin, pH 6.6] supplemented with 1 mM $MnCl_2$ and $CaCl_2$. After the column was washed with buffer A supplemented with 1.5 mM methyl α-D-mannoside to remove contaminating pigments, iodopsin was eluted with buffer A supplemented with 200 mM methyl α-D-mannoside.

To prepare nitrate-bound, gluconate-treated, or anion-unbound iodopsin samples in the CHAPS/PC system, the purified iodopsin (chloride-bound iodopsin) sample was dialyzed against buffer A containing 140 mM $NaNO_3$, 140 mM sodium gluconate, or no salt, respectively. To prepare PC liposomes containing iodopsin, the purified sample was dialyzed against buffer B [50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin, pH 6.6] with three exchanges of the buffer every 1 h and three times every 3 h. The iodopsin·NO_3, iodopsin·glu, or iodopsin·na sample in PC liposomes was then prepared by dialyzing the iodopsin sample in PC liposomes against buffer C containing 140 mM $NaNO_3$, 140 mM sodium gluconate, or no salt instead of NaCl, respectively. An equal volume of glycerol was added for UV/Vis spectral measurements from −40 to 30 °C, and two volumes of glycerol was added for measurements at liquid nitrogen temperature.

Spectrophotometry. UV/Vis absorption spectra were recorded with a Shimadzu model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. The system for the recording of absorption spectra was reported previously (*5*, *23*). An Oxford model DN-1704 cryostat was used for cooling the sample. The temperature of the sample was regulated to within 0.1 °C with a temperature controller (ITC-4, Oxford). The sample was irradiated with light from a 1-kW tungsten halogen lamp (Rikagaku Seiki) which had been passed through a glass cutoff filter (IR76 and VR68; Toshiba) or an interference filter (501 nm; Nihonshinku, and 780 nm, 700 nm; Toshiba).

Chromophore Extraction and HPLC Analysis. The experimental procedures for the chromophore extraction and HPLC analysis are identical to those reported previously (*9*, *28*). The isomeric compositions of the retinal oximes are calculated from the peak areas monitored at 360 nm using the extinction coefficients previously reported (*29*).

Calculation of Thermodynamic Parameters. The anion-unbound iodopsin in the CHAPS/PC system exhibited spectral change with an isosbestic point in the temperature range from 30 to −20 °C, indicating that it should be a mixture of two components (See Results). Because the spectrum did not change below −20 °C, where the spectrum exhibited a longer maximum, we assumed that the sample below −20 °C contains only the long-wavelength component.

The spectrum of the short-wavelength component was calculated by subtracting the spectrum of the long-wavelength component from the recorded spectrum at each temperature. The subtraction was performed so that the longer wavelength tail of the recorded spectrum originates from the long-wavelength component. The equilibrium constant was then calculated from the ratio of these two components estimated from the absorbances at 500 nm, the isosbestic point of the spectral shift upon temperature change. To estimate the difference in free energy between the long- and short-wavelength components of iodopsin, the logarithms of the equilibrium constants for the components were plotted against the inverse of the temperatures at which they were estimated, followed by fitting with a straight line by a least-squares method. Thermodynamic parameters were then calculated from the following equations:

$$\Delta G = -RT \ln K$$

$$\Delta H = -R \, \mathrm{d}(\ln K)/\mathrm{d}(1/T)$$

$$\Delta S = (-\Delta G + \Delta H)/T$$

where ΔG, ΔH, ΔS, and K are the difference free energy, the difference enthalpy, the difference entropy of the long-to-short transition, and the equilibrium constant, respectively, and R and T are the gas constant and temperature, respectively.

RESULTS

Thermal Isomerization of the Chromophore upon Warming of Bathoiodopsin in the CHAPS/PC Environment. Because of its relatively unstable nature, we were previously unable to prepare a reasonable amount of iodopsin sample binding no anion (iod·na) for spectroscopic analysis. Therefore, we prepared the iodopsin sample in the presence of gluconate ion that would be too large to bind to the anion-binding site of iodopsin, and assumed that this iodopsin sample with gluconate ion (iod·glu) should exhibit characteristics similar to iod·na. Because we recently succeeded in preparing the iod·na sample (*24*), we first compared the characteristics of iod·na with those of iod·glu. We also examined the characteristics of chloride-bound iodopsin (iod·Cl) and nitrate-bound iodopsin (iod·NO_3), which were prepared from the same iodopsin preparations. Curves 1 in Figure 1a−d are the absorption spectra under various anionic conditions of iodopsin samples solubilized with the CHAPS/PC mixture recorded at 0 °C. The absorption maximum of iod·na (curve 1 in Figure 1c) is similar to that of iod·glu (curve 1 in Figure 1d), but slightly blue-shifted and red-shifted from iod·Cl (curve 1 in Figure 1a) and iod·NO_3 (curve 1 in Figure 1b), respectively. The values are listed in Table 1.

When iod·Cl was cooled to −196 °C and irradiated with a green light, it converted to bathoiodopsin (batho·Cl) evidenced by the red shift in the absorption spectrum (inset of Figure 1a). Upon warming of the sample to −160 °C, thermal isomerization from the all-trans to 11-cis form of the chromophore occurred, resulting in conversion of batho·Cl to the original iod·Cl. Thus, the spectrum recorded after warming the sample to 0 °C (curve 2 in Figure 1a) is similar in shape to that recorded before cooling of the sample to −196 °C (curve 1 in Figure 1a). The slight decrease of absorbance in the visible region could be due to incomplete

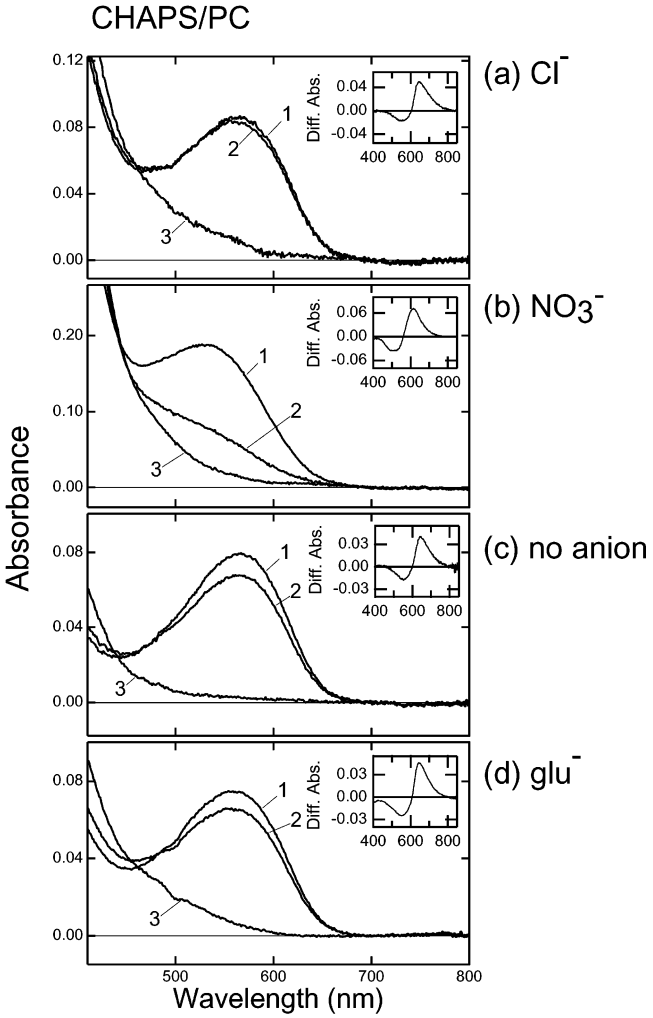

FIGURE 1: Thermal reactions of batho intermediates of iodopsin samples solubilized with a CHAPS/PC mixture. The batho intermediates are produced from (a) iod·Cl, (b) iod·NO_3, (c) iod·na, and (d) iod·glu at −196 °C. These spectra, except for iod·na, have been published previously (*26*). After the recording of the spectrum at 0 °C (curves 1), each iodopsin sample was cooled to −196 °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 iodopsin pigment in the sample (curves 3). (Inset in each panel) Difference spectrum upon irradiation to each kind of iodopsin sample at −196 °C. Positive and negative peaks represent pigment absorption after and before irradiation, respectively.

isomerization of the chromophore in batho·Cl, which causes incomplete reversion to the original iod·Cl and the formation of isopigment (iso·Cl) by the irradiation at −196 °C. The formation of isopigment was evidenced by the HPLC analysis (see below). The spectrum of iod·NO_3 after warming to 0 °C (curve 2 in Figure 1b) shows a considerable decrease of absorbance in the visible region, indicating that the chromophore of batho·NO_3 is not isomerized to an 11-cis form so that batho·NO_3 does not revert to the original iod·NO_3 but bleaches to all-*trans*-retinal and opsin through several intermediates upon warming (*25*). The spectra of iod·na and iod·glu after warming to 0 °C show a slight decrease of absorbance in the visible region. The amount of bathoiodopsins formed from the iod·na and iod·glu are similar to those from iod·Cl and iod·NO_3 (insets of Figure 1), indicating that bathoiodopsins produced from iod·na and iod·glu mainly

Table 1: Efficiency of Thermal Isomerization of Bathoiodopsin Chromophore

		λmax (nm)[a]	ratio at λmax (%)[b]
CHAPS/PC	Cl^-	573	96
	NO_3^-	538	31
	no anion	564	85
	glu^-	557	87
PC liposome	Cl^-	571	96
	NO_3^-	532	41
	no anion	532	48
	glu^-	536	58

[a] Each absorption maximum was determined from a difference spectrum recorded at 0 °C, of which baseline was recorded after the pigment was completely bleached in the presence of 10 mM NH_2OH. [b] The efficiencies are calculated as follows: The iodopsin samples are irradiated at −196 °C to produce bathoiodopsin-rich photo-steady-states, and then warmed to 0 °C. Difference spectra are calculated by subtracting the spectrum bleached completely at 0 °C as a baseline. The ratio of residual iodopsin pigment is calculated as that of absorbance at the absorption maximum of the difference spectrum.

revert to the original iodopsins through thermal isomerization of the chromophore upon warming. These results are consistent with those of a previous study and support the idea that gluconate does not bind to the anion-binding site of iodopsin (*26*). The efficiencies of thermal isomerization of the chromophores of bathoiodopsins under the various anionic conditions, which are estimated from the spectra, are listed in Table 1.

The chromophore extraction followed by HPLC analysis confirmed the occurrence of thermal isomerization in the sample of iod·Cl in CHAPS/PC (Figure 2a). Namely, 11-cis dominant chromophore composition was obtained from the iod·Cl sample before and after irradiation at −196 °C with the increase in 9-cis composition after the irradiation. Although it is clear that a considerable amount of batho·Cl, which contains all-trans chromophore (*22*), was formed by the irradiation (inset of Figure 1a), the all-trans composition was not increased in the irradiated sample. These facts are consistent with the previous observation (*9*) and indicate that all-trans chromophore in batho·Cl thermally reverts to the original 11-cis form upon warming to 0 °C. The similar trends, that is, the occurrence of thermal isomerization, were observed in the samples of iod·na and iodo·glu in CHAPS/PC (Figure 2c,d). Conversely, bathoiodopsin formed from the iod·NO_3 in CHAPS/PC bleached to all-*trans*-retinal and opsin upon warming to 0 °C, which was evidenced by the considerable increase in all-trans composition in the extracted chromophores (Figure 2b). These results are in good agreement with the spectroscopic observations shown in Figure 1 and those previously reported (*25*, *26*).

Thermal Behavior of Bathoiodopsin in the PC Liposome Environment. In the PC liposomes, the absorption maxima of iod·na and iod·glu were located at 532 and 536 nm, respectively, which are similar to that of iod·NO_3 (532 nm), but considerably different from that of iod·Cl (571 nm) (Table 1). These are in contrast to the fact that the absorption maxima of iod·na and iod·glu are located close to that of iod·Cl in the CHAPS/PC environment. Therefore, the absorption maximum of iodopsin is dependent on the environment when iodopsin has no anion in its binding site.

The reaction pathway of bathoiodopsin produced from iod·na or iod·glu is also dependent on the environment. The efficiencies of the thermal isomerization of the chromophores

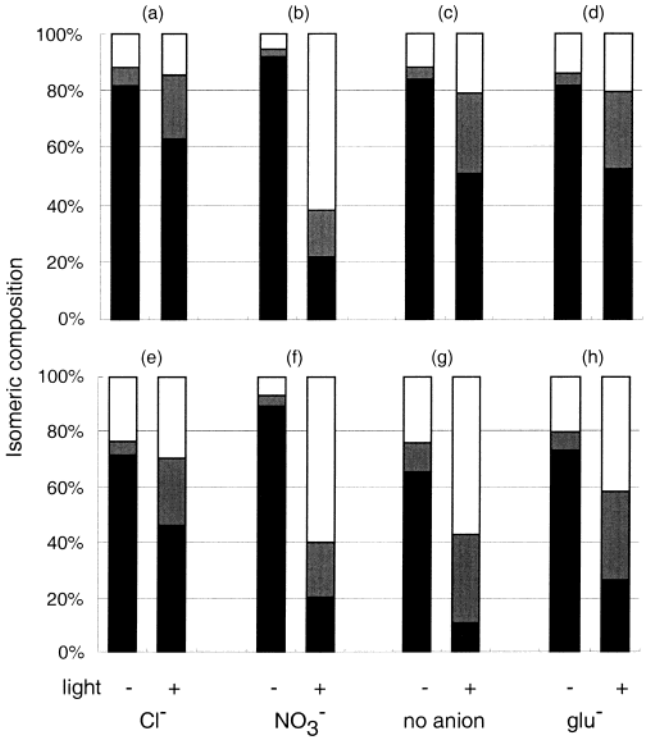

FIGURE 2: Isomeric compositions of chromophores in iodopsin samples before and after irradiation at −196 °C. Chromophores of iodopsin and its photoproducts were extracted as oximes before and after irradiation with green light (501 nm) at −196 °C until a photo-steady-state mixture were produced. The isomeric compositions of the oximes are analyzed by HPLC and are presented as percentages for the mixture of 11-cis (black), 9-cis (gray), and all-trans (white). 7-cis and 13-cis components were negligible under our experimental conditions and not included in the figures. The deviations are estimated to be within 15% based on the three independent extraction experiments from the same samples. (a) iod·Cl in CHAPS/PC mixture. (b) iod·NO_3 in CHAPS/PC mixture. (c) iod·na in CHAPS/PC mixture. (d) iod·glu in CHAPS/PC mixture. (e) iod·Cl in PC liposomes. (f) iod·NO_3 in PC liposomes. (g) iod·na in PC liposomes. (h) iod·glu in PC liposomes.

in bathoiodopsins in PC liposomes were estimated under various anionic conditions as were done in the CHAPS/PC environment (Table 1). The results showed that only batho·Cl exhibits efficient thermal isomerization in PC liposomes. The amounts of the bleached pigments in PC liposomes are almost the same among batho·NO_3, batho·na, and batho·glu, although it was slightly lower for batho·glu.

Chromophore extraction followed by HPLC analysis also confirmed the reaction characteristics of bathoiodopsins in these samples (Figure 2e−h), although the amount of all-trans form in the extracted chromophore, which could be formed by the thermal isomerization during the extraction procedure, was slightly increased. Namely, the all-trans chromophores were significantly increased after irradiation at −196 °C followed by warming to 0 °C in iod·NO_3, iod·na, and iod·glu (Figure 2f−h), while it was scarcely changed in iod·Cl (Figure 2e).

These results clearly show that in iod·na and iod·glu, the absorption maxima and extent of thermal isomerization of the chromophore are dependent on the environment, while in iod·Cl and iod·NO_3, they are not. In other words, the properties of iodopsin are dependent on the environment when the anion-binding site of iodopsin is vacant, but not when it is occupied by chloride or nitrate. Iod·na and iod·

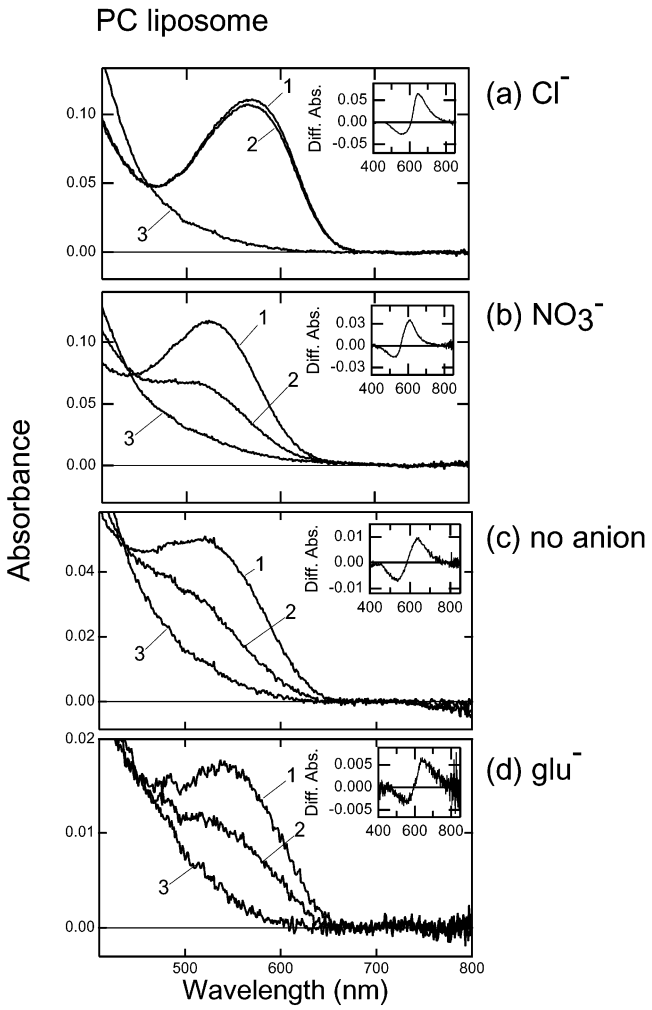

FIGURE 3: Thermal reactions of batho intermediates of iodopsin samples in PC liposome. The batho intermediates were produced from (a) iod·Cl, (b) iod·NO_3, (c) iod·na, and (d) iod·glu at −196 °C. The spectra were recorded in the same manner as in Figure 1. (Inset in each panel) Difference spectrum upon irradiation to each kind of iodopsin sample at −196 °C. Positive and negative peaks represent pigment absorption after and before irradiation, respectively.

glu behaved like iod·Cl in the CHAPS/PC mixture, while they behaved like iod·NO_3 in PC liposomes. Thus, it is of interest to investigate the mechanism leading to the different characteristics of iodopsin.

Temperature Effect on the Spectral Features of Iodopsin. In addition to the environmental dependence, iod·na and iod·glu show temperature-dependent absorption spectra in CHAPS/PC (Figure 4). In fact, these pigments underwent a change in their absorption maxima from ca. 560 to ca. 520 nm upon warming from −10 to 30 °C. On the other hand, iod·Cl and iod·NO_3 showed little change in their maxima upon the temperature change. These results also suggest that the protein moiety of iodopsin becomes flexible when it has no anion in the binding site.

Temperature-dependent spectral shifts were also observed in the PC liposome environment (Figure 5). Like in the CHAPS/PC environment, absorption maxima of iod·Cl and iod·NO_3 hardly changed, while those of iod·na and iod·glu changed in PC liposomes. A noteworthy point is that the shifts of maxima upon temperature change in iod·na and iod·glu are much smaller than those observed in the CHAPS/PC system. These results suggest that PC liposome has some role in inhibiting the flexibility of the protein moiety of iodopsin. This tendency is similar to that of the thermal reaction of bathoiodopsin that has been shown in the present study.

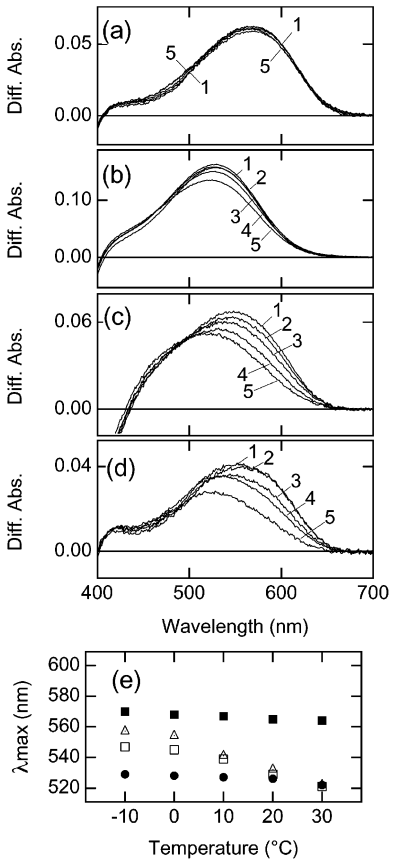

FIGURE 4: Temperature dependence of the absorption spectrum of iodopsin samples in the CHAPS/PC mixture. Absorption spectra of (a) iod·Cl, (b) iod·NO_3, (c) iod·na, and (d) iod·glu were recorded at −10, 0, 10, 20, and 30 °C (curves 1−5). The baseline of each spectrum is the spectrum obtained after each sample was completely bleached in the presence of 10 mM NH_2OH. (e) Shifts of absorption maxima in samples of iod·Cl (closed squares), iod·NO_3 (closed circles), iod·na (open squares), and iod·glu (open triangles) are plotted as a function of temperature.

As shown in Figure 4, the absorption spectrum of iod·na shifts to a longer wavelength with an isosbestic point at 500 nm upon cooling. These results strongly suggest that iod·na has two conformations with different absorption maxima and these two form a temperature-dependent equilibrium. The absorption spectrum of iod·na did not change below −20 °C, and therefore, we assumed that iod·na at −20 °C should be a single conformation having a longer absorption maximum. We referred to this component as the long-wavelength component (LWC). The form having the shorter absorption maximum (short-wavelength component, SWC) was calculated by subtracting the spectrum of the LWC from the spectrum recorded at each temperature. The subtraction was performed so that the longer-wavelength tail of the recorded spectrum originates from the LWC. It should be noted that the absorption maxima of LWC and SWC are almost the same as those of iod·Cl and iod·NO_3, respectively, indicating that chloride and nitrate stabilize each of the conformations existing in the absence of anion.

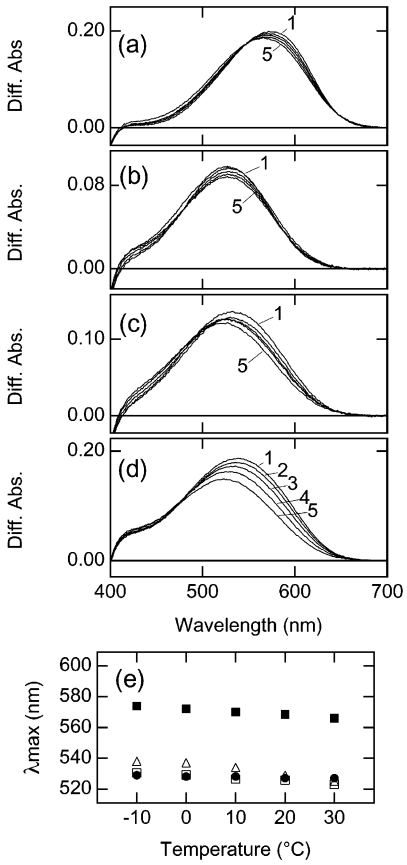

FIGURE 5: Temperature dependence of the absorption spectrum of iodopsin samples in PC liposome. Absorption spectra of (a) iod·Cl, (b) iod·NO_3, (c) iod·na, and (d) iod·glu were recorded at −10, 0, 10, 20, and 30 °C (curves 1−5). The spectra were recorded in the same manner as in Figure 4. (e) Shifts of absorption maxima in samples of iod·Cl (closed squares), iod·NO_3 (closed circles), iod·na (open squares), and iod·glu (open triangles) are plotted as a function of temperature.

To estimate the thermodynamic parameters, we plotted the equilibrium constants as a function of the inverse of the temperature at which they were estimated, and fitted a straight line using a least-squares method. The ΔH between the two components was calculated to be 13 kcal/mol, while the ΔS was calculated to be 43.7 cal/mol/deg. Both the enthalpy and entropy of the LWC are smaller than those of the SWC, suggesting a tighter structure for the LWC than SWC.

DISCUSSION

In the present study, we have examined the effects of anion on the visible absorption spectrum of iodopsin and the thermal isomerization of the chromophore in bathoiodopsin under different anionic and environmental conditions. The results showed that iodopsin is likely to be present in two states that form a temperature and environment-dependent equilibrium. In addition, one of the forms exhibits a longer absorption maximum and thermal isomerization reaction in the batho-intermediate similar to iod·Cl, while the other is similar to iod·NO_3. Thus, chloride and nitrate could stabilize each of the forms of iodopsin, when they bind to the binding site. On the basis of the present results and the FTIR analyses of iodopsins and bathoiodopsins we have recently reported (*23*, *24*), we discuss the effect of anion binding on the local structure of iodopsin and the structure of the chromophore in bathoiodopsin.

Effect of Anion-Binding on the Two Forms of Iodopsin. In the CHAPS/PC environment, batho·na showed thermal isomerization of the chromophore like batho·glu and batho·Cl do. These results are consistent with previously published results (*26*), which implied a special effect of nitrate on the thermal isomerization reaction of bathoiodopsin. On the other hand, in the PC liposome environment, no bathoiodopsins other than batho·Cl showed an isomerization reaction upon warming. These results support those of recently published FTIR experiments, in which batho·Cl had a chromophore−opsin interaction different from that of batho·na or batho·NO_3, while the latter two showed a similar interaction (*24*). In the present study, we have found that iodopsin having no anion in its binding site is likely to be composed of two forms, LWC and SWC, which are stabilized by the binding of chloride and nitrate, respectively, in the CHAPS/PC environment. Therefore, it is reasonable to hypothesize that the bathoiodopsin produced from the LWC shows thermal isomerization, while that from the SWC does not show trans-to-cis isomerization but undergoes a normal bleaching process. This hypothesis explains the relationship between the effect of anion on the absorption spectrum of iodopsin and on the thermal isomerization reaction of bathoiodopsin in both the CHAPS/PC and PC liposome systems as follows: Almost all the iod·na and iod·glu would be composed of LWC at −196 °C since it is clear that iod·na and iod·glu in the CHAPS/PC environment show a large red-shift upon cooling to the liquid nitrogen temperature. Thus, it is reasonable that bathoiodopsin formed from iod·na and iod·glu shows thermal isomerization in the CHAPS/PC environment. On the other hand, in the PC liposome environment, iod·na and iod·glu showed absorption spectra similar to that of iod·NO_3 at 0 °C. In addition, iod·na in PC liposomes exhibited a maximum at 534 nm even at −196 °C. The temperature-dependent shift of the absorption maximum in iod·glu in the PC liposome environment is also significantly smaller than that observed in the CHAPS/PC environment. Therefore, it is likely that iod·na and iod·glu in PC liposomes have mainly a single conformation (SWC) even at −196 °C. This is consistent with our observation that batho·na and batho·glu showed thermal behavior similar to that of batho·NO_3 in this environment. Therefore, on the basis of the two structures of bathoiodopsin observed in FTIR spectra recorded in the PC liposome environment (*24*), iod·Cl would be in an LWC, while iod·NO_3, iod·na, and iod·glu would be in an SWC.

Mechanism of the Thermal Isomerization Reaction of Bathoiodopsin. We have recently examined the effect of anions on the local structure of the chromophore and the protein moiety of iodopsin by means of FTIR spectroscopy. The batho/iod difference FTIR spectrum obtained from iod·Cl showed features different from the spectra for iod·NO_3 and iod·na, while the features of the latter two were almost identical (*24*). The most prominent difference is seen in the 830 cm^{-1} band of the spectrum of bathoiodopsin. This band, which originated from the C_{14}−HOOP mode of the chromophore (*23*), has great intensity in the FTIR spectrum of batho·Cl. The intensity of this band is largely diminished in the spectra of the other two kinds of bathoiodopsin, and this is also the case in our preliminary study on batho·glu (Hirano

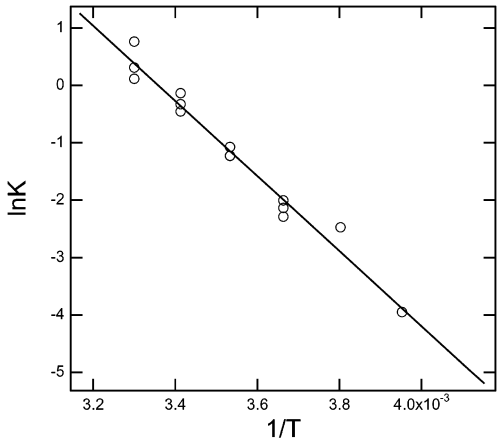

FIGURE 6: Temperature dependence of the equilibrium constants between the long-wavelength component (LWC) and the short-wavelength component (SWC) of iodopsin·na in CHAPS/PC. Equilibrium constants were plotted against the inverse of the temperatures at which they were estimated in a van't Hoff manner. See the materials and methods section for details.

et al., unpublished data). Since the appearance of this mode is due to the distortion of the polyene chain near the C_{14} position of the chromophore, one can speculate that the distorted structure near the C_{14} position would be a driving force for the thermal isomerization of the chromophore in bathoiodopsin. Thus, one question arises from these considerations: what would make the chromophore of bathoiodopsin distorted at C_{14}?

One possibility is a steric interaction between some portion of the photoisomerized chromophore and nearby residue(s), and this interaction forces the C_{14} position of the chromophore to become distorted. This steric interaction could interfere with the complete cis−trans isomerization of the chromophore (*30*−*32*). In fact, according to the 3D structure of bovine rhodopsin reported recently (*33*, *34*), some residues, Y268 for instance, locate within 5 Å of both the chromophore and IV−V loop region, the latter of which contains the anion-binding site of iodopsin. Therefore, binding of chloride would induce the relocation of the anion-binding site and nearby residues, thereby resulting in the change of the interaction between the chromophore and surrounding residues. In this context, the present results can be summarized by the scheme presented in Figure 7. The iod·na in the CHAPS/PC environment is composed of two conformations, LWC and SWC, which form an equilibrium state. LWC is stabilized by the binding of chloride, while SWC is stabilized by the binding of nitrate in the CHAPS/PC environment. In PC liposome environment, the equilibrium tends to favor SWC in the absence of anion, and only the binding of chloride stabilizes LWC. The structural change around the chloride-binding site induces relocation of the residues near the chromophore. LWC should have a tighter structure than SWC, which is suggested by the thermodynamic parameters between the two.

When the LWC is irradiated at −196 °C, some portion (one candidate is the 13-methyl group) of the photoisomerized chromophore sterically interacts with nearby residue(s), and this steric interaction should bring about the distorted structure around C_{14} of the chromophore in addition to the C_{11} portion. This distorted structure around C_{14} would hinder the normal relaxation process of the torsion at C_{11} and the

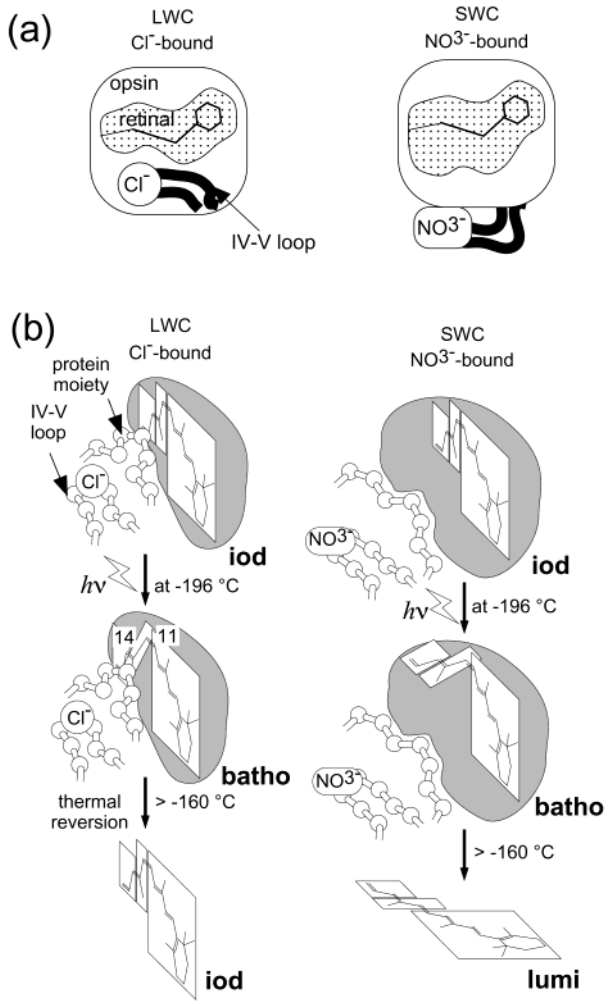

FIGURE 7: Schematic drawing of the thermal reverse reaction of bathoiodopsin. (a) Iodopsin has two conformations, and each of them is stabilized by binding of NO_3^- (SWC) or Cl^- (LWC). The retinal-binding pocket (shadowed areas) of LWC should be smaller than that of SWC, as suggested from the thermodynamic analysis. In the case that no anion is bound to iodopsin, the structure of the anion-binding site is flexible, resulting in equilibrium between the structures of SWC and LWC. (b) The retinal-binding pocket (shadowed areas), especially around C_{14}, of LWC is smaller than that of SWC. Bathoiodopsin produced from LWC at −196 °C has its chromophore distorted around C_{14} because of the steric interaction with nearby residue(s), and thereby thermally reverts to the original state (iodopsin). On the other hand, bathoiodopsin produced from SWC at −196 °C does not have this steric interaction and the distorted structure around C_{14} of the chromophore, and thereby converts to the next intermediate, lumiiodopsin.

movement of the β-ionone ring portion of the chromophore that is necessary for batho-to-lumi transition (*35*, *36*). Thus, bathoiodopsin produced at −196 °C thermally reverts to the original iodopsin above −160 °C. When SWC is irradiated, there is little torsion around C_{14} of the photoisomerized chromophore because of the structural change of the protein moiety around the chromophore. This structure modifies the twisting structure around C_{11}, and bathoiodopsin produced at −196 °C converts to lumiiodopsin above −160 °C and shows a normal decay.

The present study has revealed the relationship between the structure and the thermal behavior of the batho intermediate produced from two conformations of iodopsin. The relationship indicated that the distorted structure around C_{14}

of the all-trans chromophore is strictly correlated to the thermal isomerization of the chromophore in bathoiodopsin. Thus, it is our next research to determine what part of the protein moiety gives the distorted structure around C_{14} of the chromophore, and how the anion-binding site is related to such a structural change. Elucidation of these issues will lead to a better understanding of the molecular mechanism of the thermal isomerization of the chromophore in bathoiodopsin.

ACKNOWLEDGMENT

We thank Drs. Y. Imamoto, S. Tachibanaki, A. Terakita, and T. Tamotsu, and Mr. T. Morizumi for cooperation in preparing the iodopsin samples. We also express great thanks to Drs. H. Kandori and M. Ishiguro for valuable discussions.

REFERENCES

1. Wald, G., Brown, P. K., and Smith, P. H. (1955) *J. Gen. Physiol. 38*, 623−680.
2. Kuwata, O., Imamoto, Y., Okano, T., Kokame, K., Kojima, D., Matsumoto, H., Morodome, A., Fukada, Y., Shichida, Y., Yasuda, K., Shimura, Y., and Yoshizawa, T. (1990) *FEBS Lett. 272*, 128−32.
3. Okano, T., Kojima, D., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5932−6.
4. Tokunaga, F., Iwasa, T., Miyagishi, M., and Kayada, S. (1990) *Biochem. Biophys. Res. Commun. 173*, 1212−7.
5. Imai, H., Terakita, A., Tachibanaki, S., Imamoto, Y., Yoshizawa, T., and Shichida, Y. (1997) *Biochemistry 36*, 12773−9.
6. Okada, T., Matsuda, T., Kandori, H., Fukada, Y., Yoshizawa, T., and Shichida, Y. (1994) *Biochemistry 33*, 4940−6.
7. Yoshizawa, T., and Wald, G. (1967) *Nature 214*, 566−71.
8. Kandori, H., Mizukami, T., Okada, T., Imamoto, Y., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8908−12.
9. Imamoto, Y., Yoshizawa, T., and Shichida, Y. (1996) *Biochemistry 35*, 14599−607.
10. Shichida, Y., Okada, T., Kandori, H., Fukada, Y., and Yoshizawa, T. (1993) *Biochemistry 32*, 10832−8.
11. Matsumoto, H., Tokunaga, F., and Yoshizawa, T. (1975) *Biochim. Biophys. Acta 404*, 300−8.
12. Crescitelli, F. (1978) *Vision Res. 18*, 1421−2.
13. Knowles, A. (1976) *Biochem. Biophys. Res. Commun. 73*, 56−62.
14. Fager, L. Y., and Fager, R. S. (1979) *Exp. Eye Res. 29*, 401−8.
15. Shichida, Y., Kato, T., Sasayama, S., Fukada, Y., and Yoshizawa, T. (1990) *Biochemistry 29*, 5843−8.
16. Kleinschmidt, J., and Harosi, F. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9181−5.
17. David-Gray, Z. K., Cooper, H. M., Janssen, J. W., Nevo, E., and Foster, R. G. (1999) *FEBS Lett. 461*, 343−7.
18. Yokoyama, S., and Radlwimmer, F. B. (1999) *Genetics 153*, 919−32.
19. Sun, H., Macke, J. P., and Nathans, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8860−5.
20. Radlwimmer, F. B., and Yokoyama, S. (1998) *Gene 218*, 103−9.
21. Yokoyama, S., and Radlwimmer, F. B. (1998) *Mol. Biol. Evol. 15*, 560−7.
22. Lin, S. W., Imamoto, Y., Fukada, Y., Shichida, Y., Yoshizawa, T., and Mathies, R. A. (1994) *Biochemistry 33*, 2151−60.
23. Imamoto, Y., Hirano, T., Imai, H., Kandori, H., Maeda, A., Yoshizawa, T., Groesbeek, M., Lugtenburg, J., and Shichida, Y. (1999) *Biochemistry 38*, 11749−54.
24. Hirano, T., Imai, H., Kandori, H., and Shichida, Y. (2001) *Biochemistry 40*, 1385−92.
25. Imamoto, Y., Kandori, H., Okano, T., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1989) *Biochemistry 28*, 9412−6.
26. Tachibanaki, S., Imamoto, Y., Imai, H., and Shichida, Y. (1995) *Biochemistry 34*, 13170−5.
27. Okano, T., Fukada, Y., Artamonov, I. D., and Yoshizawa, T. (1989) *Biochemistry 28*, 8848−56.
28. Imai, H., Hirano, T., Terakita, A., Shichida, Y., Muthyala, R. S., Chen, R. L., Colmenares, L. U., and Liu, R. S. (1999) *Photochem. Photobiol. 70*, 111−5.
29. Trehan, A., Liu, R. S. H., Shichida, Y., Imamoto, Y., Nakamura, K., and Yoshizawa, T. (1990) *Bioorg. Chem. 18*, 30−40.
30. Yoshizawa, T., and Wald, G. (1963) *Nature 197*, 1279−86.
31. Eyring, G., Curry, B., Mathies, R., Fransen, R., Palings, I., and Lugtenburg, J. (1980) *Biochemistry 19*, 2410−8.
32. Eyring, G., Curry, B., Broek, A., Lugtenburg, J., and Mathies, R. (1982) *Biochemistry 21*, 384−93.
33. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science 289*, 739−45.
34. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 5982−7.
35. Borhan, B., Souto, M. L., Imai, H., Shichida, Y., and Nakanishi, K. (2000) *Science 288*, 2209−12.
36. Okada, T., Kandori, H., Shichida, Y., Yoshizawa, T., Denny, M., Zhang, B. W., Asato, A. E., and Liu, R. S. (1991) *Biochemistry 30*, 4796−802.

BI034671W