Purification and Low Temperature Spectroscopy of Gecko Visual Pigments Green and Blue[†]

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ABSTRACT: We purified two kinds of visual pigments, gecko green and gecko blue, from retinas of Tokay geckos (*Gekko gekko*) by two steps of column chromatography, and investigated their photobleaching processes by means of low temperature spectroscopy. Absorption maxima of gecko green and blue solubilized in a mixture of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and phosphatidylcholine were 522 and 465 nm, respectively, which are close to those observed in the photoreceptor cells. Low temperature spectroscopy identified six intermediates in the photobleaching process of gecko green; batho (λ_{max} = 569 nm), BL (λ_{max} = 519 nm), lumi (507 nm), meta I (~486 nm), meta II (~384 nm), and meta III intermediates (~500 nm). In contrast to the high similarity in amino acid sequence between gecko green and iodopsin [Kojima, D., et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 6841−6845], the batho-green did not revert thermally to original gecko green but converts to the next intermediate. The photobleaching process of gecko blue was investigated by low temperature spectroscopy, and three intermediates, meta I (λ_{max} = ~470 nm), meta II (λ_{max} = ~370 nm) and meta III (λ_{max} = ~475 nm), were identified. A comparative study on the thermal behavior of meta intermediates revealed that the thermal stability of meta II intermediate of both of the gecko visual pigments is lower than that of metarhodopsin II. The result supports the idea that both the gecko visual pigments are cone-type ones.

A nocturnal lizard, Tokay gecko (*Gekko gekko*) has two kinds of visual pigments in the retina. The major one is a green-sensitive pigment, gecko green (λ_{max}[1] = 521 nm), and the minor one is a blue-sensitive pigment, gecko blue (λ_{max} = 467 nm). In spite of the pure-rod morphology of the photoreceptor cells, the amino acid sequences of gecko green and blue are more similar to cone visual pigments than to rod visual pigments, rhodopsins (Kojima et al., 1992). Gecko green is the most similar (82.6% identity) to a chicken cone pigment, iodopsin (Kuwata et al., 1990; Tokunaga et al., 1990), and they form a family of long-wavelength-sensitive visual pigments, group L (Okano et al., 1992). On the other hand, gecko blue is the most similar (82.3% identity) to another chicken cone pigment, chicken green (Okano et al., 1992; Wang et al., 1992), although gecko blue displays relatively high similarities (more than 70% identities) to vertebrate rhodopsins, rather than to the other cone pigments (less than 55%) such as iodopsin, chicken blue, and chicken violet. Therefore, it is of interest to investigate whether gecko visual pigments present in rod-shaped photoreceptor cells behave like cone visual pigments.

The biochemical properties of gecko visual pigments were elucidated by the pioneering works of the late Prof. Crescitelli. According to his works, gecko green displays some characteristics similar to those of iodopsin; its spectrum is blue-shifted by the depletion of chloride (chloride effect; Crescitelli, 1977a), its opsin moiety rapidly regenerates upon addition of 11-*cis*-retinal (Crescitelli, 1979), and it is unstable against hydroxylamine in the dark (Crescitelli, 1963). Furthermore, he showed that the other pigment, gecko blue, has some biochemical properties similar to those of rhodopsin; it is insensitive to hydroxylamine in the dark and displays no chloride effect (Crescitelli, 1980). The similarity of biochemical properties between gecko green and iodopsin is consistent with homology in amino acid sequence between them (Kojima et al., 1992), and the absence of the chloride effect in gecko blue is consistent with the fact that chicken green and rhodopsins display no chloride effects. It should be noted that, however, stability of gecko blue against hydroxylamine is in contrast to the high sequence similarity to chicken green, which is unstable against hydroxylamine (Fager & Fager, 1979; Okano et al., 1989). This inconsistency suggests that some properties of visual pigments are not necessarily derived from overall similarities in amino acid sequences. In fact, our recent work on the comparative

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[1] Abbreviations: BL, intermediate between batho and lumi intermediates; BSI, blue-shifted-intermediate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ConA, concanavalin A; DTT, dithiothreitol; HEPES, N-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; λ_{max}, absorption maximum (maxima); PC, L-α-phosphatidylcholine from fresh egg yolk; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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studies between chicken green and rhodopsin (Shichida et al., 1994) shows that chicken green displays some of molecular properties clearly different from those of rhodopsin, although their amino acid identities are 72.4% (Okano et al., 1992).

The present paper is devoted to detailed characterization of the spectroscopic properties of gecko visual pigments in order to elucidate whether gecko visual pigments have photobleaching processes similar to those of their sequence homologues (iodopsin and chicken green) or to that of rhodopsin. One of the interesting phenomena observed in the photobleaching process of iodopsin is that its batho intermediate (bathoiodopsin) produced at liquid nitrogen temperature does not thermally bleach to *all-trans*-retinal and its protein moiety, but reverts to the original iodopsin (Yoshizawa & Wald, 1967). Since the reversal reaction of batho intermediate at low temperature is not observed in rhodopsin and this reaction is suppressed when chloride bound to iodopsin is replaced by nitrate, a relationship between the reversal reaction and the chloride binding has been suggested (Imamoto et al., 1989). Furthermore, iodopsin has a BL intermediate whose spectral properties are different from those of BL(BSI) intermediate of rhodopsin (Shichida et al., 1993). Therefore, we have identified batho and BL intermediates of gecko green, with special attention to the thermal behavior of batho intermediate and the spectral properties of the BL intermediate, to see whether these properties are common to the group L visual pigments having a chloride binding site (Kleinschmidt & Harosi, 1992; Wang et al., 1993).

Another purpose in this study is to identify meta intermediates of gecko green and blue. Recent studies on iodopsin (Shichida et al., 1993; Okada et al., 1994) and chicken green (Shichida et al., 1994) showed that both visual pigments have meta I and meta II intermediates in their bleaching processes, while the meta III intermediate is observed only in chicken green. Since the physiological function of the meta III intermediate in rhodopsin is thought to extend the survival of the physiologically active intermediate, meta II intermediate, by forming an equilibrium mixture with meta II intermediate (Kibelbek et al., 1991), it is of interest to investigate whether or not gecko visual pigments have meta III intermediates in their bleaching processes. Furthermore, investigations of the thermal behavior of meta intermediates, especially meta II intermediate, are important to get a clue to the signal transduction mechanism in gecko photoreceptor cells. Therefore, we have tried to identify meta intermediates with special attention to their thermal behavior.

MATERIALS AND METHODS

Chemicals. Aprotinin, leupeptin, *all-trans*-retinal, and L-α-phosphatidylcholine (PC) from egg yolk (type XI-E) were products of Sigma. ConA-Sepharose and CM-Sepharose FF were purchased from Pharmacia LKB Biotechnology Inc. 11-*cis*-Retinal was purified by means of HPLC according to Maeda et al. (1978) and stored at −80 °C until use.

Buffers. The pH of all the buffers below were adjusted to 6.6 at 4 °C. Buffer P contained 50 mM HEPES, 140 mM NaCl, 1 mM DTT, 50 kallikrein inhibitor units/mL aprotinin, and 4 mg/mL leupeptin. Buffer E was the same as buffer P except for containing 0.75% CHAPS, 1.0 mg/mL PC, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. Buffer A-140 was the same as buffer P except for containing 0.6% CHAPS, 0.8 mg/mL PC, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. Buffer A-10 was the same as buffer A-140, except for 10 mM NaCl. Buffer D-20 contained 20% (w/v) glycerol, 0.6% CHAPS, 0.8 mg/mL PC, 50 mM HEPES, 1 mM DTT, 50 kallikrein inhibitor units/mL aprotinin, and 4 mg/mL leupeptin.

Isolation of Gecko Retinas. All operations were carried out in complete darkness or under dim red light (>660 nm). Tokay geckos (*G. gekko*) were dark-adapted overnight. All procedures described below were performed at 4 °C unless otherwise stated. The dissected eyes were placed in 4% potassium alum for 1 h. The retinas were then removed from the eyes and placed in distilled water. After placing the retinas in 4% potassium alum for 30 min, they were washed twice with distilled water and placed in standard phosphate buffer (pH 7.4). They were frozen and kept at −80 °C until use.

Purification of Visual Pigments. Visual pigments were purified from gecko retinas according to the method of Okano et al. (1989) with some modifications. Sixty-nine of the frozen retinas were thawed and pelleted by centrifugation (20000*g*, 15 min). The pelleted retinas were suspended with buffer E (approximately 0.8 mL per retina) containing 2-fold molar excess (0.7 OD of absorbance at 380 nm) of 11-*cis*-retinal over the amount (estimated absorbance at 520 nm, 0.7 OD) of opsin moiety of dominant visual pigment (gecko green) and homogenized with a Teflon homogenizer (30 strokes). After centrifugation (110000*g*, 1 h), the supernatant was collected. Buffer P (15 mL) supplemented with 1 mM $MnCl_2$ and $CaCl_2$ was added to the supernatant (61 mL), in order to decrease the concentrations of CHAPS and PC to 0.6% and 0.8 mg/mL, respectively.

The diluted supernatant termed "CHAPS-PC extract" (76 mL) was loaded on a ConA-Sepharose affinity column (10 mm × 85 mm) which had been equilibrated with 15 bed volumes of buffer A-140 at a flow rate of 12 mL/h. After loading, the column was successively washed with 8 bed volumes of buffer A-140 and with 10 bed volumes of buffer A-10 to remove unbound materials. A mixture containing mainly gecko green was eluted in order with 47 bed volumes of 1.5 mM methyl α-mannoside in buffer A-10 (fractions 1−13) and with 13 bed volumes of 5 mM methyl α-mannoside in buffer A-10 (fractions 14−17). Then, a mixture containing mainly gecko blue was eluted with 16 bed volumes of 200 mM methyl α-mannoside in buffer A-10 (fractions 18−22). Finally, the proteins still bound to the column were eluted with 11 bed volumes of 500 mM methyl α-mannoside in buffer A-10 without $MnCl_2$ and $CaCl_2$ (fractions 23 and 24). Throughout these procedures (loading, washing, and elution), the flow rate was fixed at 8.5 mL/h and the absorbance of the eluate was continuously monitored at 280 nm. Glycerol was added to the eluate to give the final concentration of 20% (w/v) in order to stabilize the visual pigments.

A part of the eluates with 1.5 and 5 mM methyl α-mannoside (fractions 2−17) containing mainly gecko green were mixed together and applied to a CM-Sepharose column (16 mm × 135 mm) which had been equilibrated with 13 bed volumes of 10 mM NaCl in buffer D-20. After the column was washed with 5 bed volumes of 10 mM NaCl in buffer D-20, gecko green was eluted with 140 mM NaCl in buffer D-20 at a flow rate of 40 mL/h.

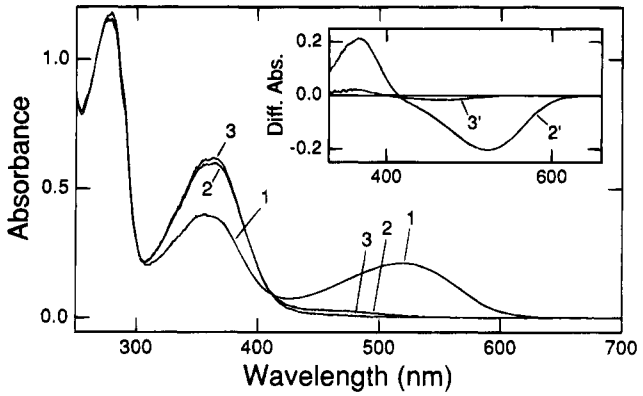

FIGURE 1: Absorption spectra of the CHAPS-PC extract of gecko retinas. In the presence of 50 mM hydroxylamine, an aliquot of the CHAPS-PC extract of gecko retinas (curve 1) was irradiated with a red light (>610 nm; curve 2), which bleached only the green-sensitive visual pigment, gecko green. Subsequent irradiation with a yellow light (>500 nm; curve 3) bleached the blue-sensitive visual pigment, gecko blue. (Inset) Difference spectra calculated from curves 1 and 2 (curve 2′) and from curves 2 and 3 (curve 3′).

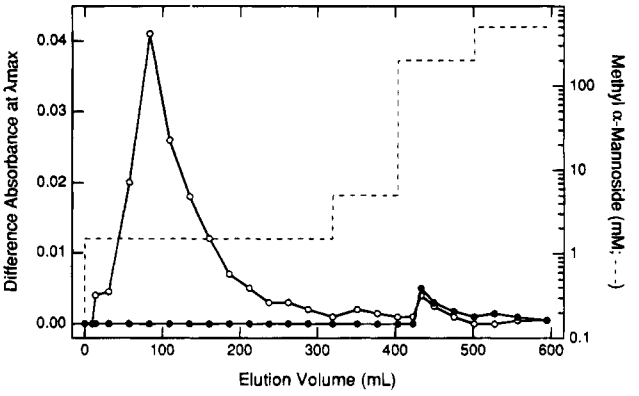

FIGURE 2: Elution profile of gecko green and blue from ConA-Sepharose column. The CHAPS-PC extract of gecko retinas was adsorbed on a ConA-Sepharose column, from which the visual pigments were sequentially eluted with 1.5, 5, 200, and 500 mM methyl α-mannoside (dashed line) in buffer A-10. Difference absorbances at λ_{max} of gecko green (open circles) and blue (closed circles) in each fraction were estimated by the partial bleaching method (see Materials and Methods).

On the other hand, the fractions eluted with 200 mM methyl α-mannoside from the ConA column (fractions 18−22) were applied on another CM-column, which had been equilibrated with 13 bed volumes of buffer D-20 without NaCl. After the column was washed with 5 bed volumes of 10 mM NaCl in buffer D-20, gecko blue was eluted with 140 mM NaCl in buffer D-20 at a flow rate of 28.5 mL/h.

To prepare the gecko green sample for low temperature spectroscopy, a fraction containing the most concentrated gecko green eluted from the CM-Sepharose column was dialyzed against buffer D-20 with 140 mM NaCl for 1 day in complete darkness at 4 °C with several renewals of the buffer, and glycerol was added to give a final concentration of 71% (v/v). If necessary, the fraction was further concentrated (about 4-fold) using a ultrafiltration membrane (AMICON, YM30) before the dialysis. The gecko blue sample for low temperature spectroscopy was prepared in a manner similar to that described above, from a fraction eluted from the CM-Sepharose column chromatography that had the highest ratio of the gecko blue/green.

Chicken rhodopsin for low temperature spectroscopy was isolated from chicken retinas and purified by the method reported by Okano et al. (1989). The concentrations of detergent and glycerol in the chicken rhodopsin sample were identical with those of gecko green and blue samples.

Spectroscopy. In the course of purification of gecko visual pigments, absorption spectra of the eluted fractions were recorded with a Shimadzu Model MPS-2000 spectrophotometer, from which the data were transferred to an NEC PC-9801RA personal computer. The sample in an optical cell (volume, 0.4 mL; width, 2 mm; light path, 1 cm) was kept at 0 °C by using a thermostated cell holder equipped in the spectrophotometer. The amount of each visual pigment in the sample was estimated by the partial bleaching method. A solution of 1 M hydroxylamine hydrochloride (pH 6.6; neutralized with NaOH) was added to each sample at 0 °C, to give the final concentration of 50 mM. Each sample was irradiated first with a red light (>610 nm), which bleached only gecko green. Then the sample was irradiated with an yellow light (>500 nm) until gecko blue was completely bleached. The light source used for irradiation of the sample was a 1-kW tungsten-halogen lamp (Rikagaku Co. Ltd.). Wavelengths of the irradiation light were selected by a cutoff filter (Toshiba Co. Ltd., VR63 or VY52). To remove heat from the irradiation light, a 5-cm water layer was placed between the light source and a filter(s).

For low temperature spectroscopy, a glass optical cryostat (Yoshizawa & Shichida, 1982) or a continuous flow cryostat (Oxford, CF1204) was installed in the sample compartment of the spectrophotometer to keep the sample at low temperatures. The sample was irradiated with a colored light at wavelengths selected by a cutoff filter [Toshiba Co. Ltd.; VR69 (transmitting light >670 nm), VR63 (>610 nm), VR60 (>580 nm), VO59 (>570 nm), VO58 (>560 nm), VO55 (>530 nm), VO54 (>520 nm) or VY52 (>500 nm)] or an interference filter (Nihonshinku, 501 nm; half bandwidth = 2 nm). To obtain a required intensity of light, a neutral density filter (Toshiba Co. Ltd., TND25) was also used.

Some of the data recorded were subjected to calculations (smoothing, normalization, or fitting) using a Macintosh Quadra 650 equipped with Igor software.

RESULTS

Purification of Gecko Visual Pigment. Curve 1 in Figure 1 shows the absorption spectrum of CHAPS-PC extract of gecko retinas in the presence of 50 mM hydroxylamine at 0 °C. The relative content of each visual pigment in the sample was estimated by successive irradiation with >610- and >500-nm light (curves 2 and 3 in Figure 1). The difference spectra between the spectra recorded before and after each irradiation are shown in the inset of the Figure 1, indicating that the ratio of gecko green (λ_{max} = 522 nm)/gecko blue (λ_{max} = 465 nm) was about 10. The extract was applied to a ConA-Sepharose column, from which gecko green and blue were selectively eluted by increasing the concentration of methyl α-mannoside in the elution buffer (Figure 2). Fractions eluted with 1.5 and 5 mM methyl α-mannoside contained only gecko green, while fractions eluted with 200 mM methyl α-mannoside were composed of similar amounts of gecko green and blue. The recoveries from the column were about 80% for gecko green and 60% for gecko blue. Though UV-sensitive photoreceptor cells have been recently

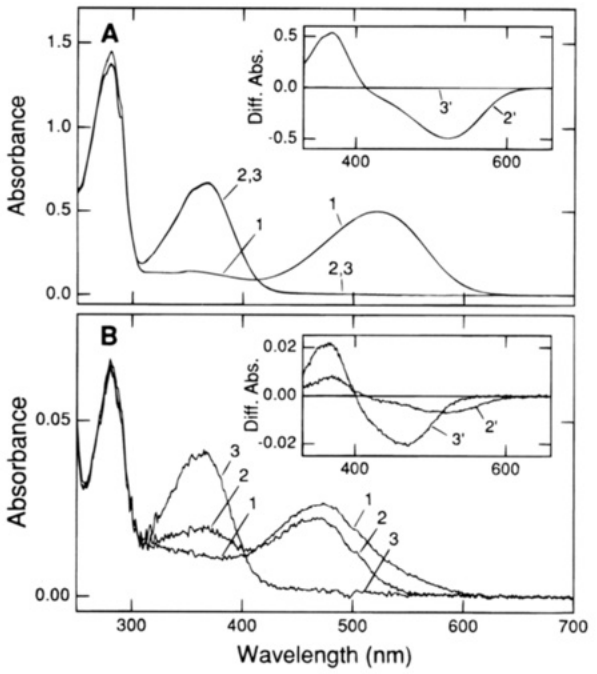

FIGURE 3: Absorption spectra of gecko visual pigments eluted from CM-Sepharose columns. Curves 1 in panels A and B are the absorption spectra of the fractions containing only gecko green (panel A) and mainly gecko blue (panel B), respectively, in the presence of 50 mM hydroxylamine. Each fraction was subsequently irradiated with a red light (>610 nm; curves 2) and a yellow light (>500 nm; curves 3), for estimating the percentage of each visual pigment contained in the fraction. (Inset) Difference spectra calculated from curves 1 and 2 (curve 2′) and from curves 2 and 3 (curves 3′).

found in the gecko retina (Loew, 1994), we were not able to detect the UV-sensitive visual pigment in our preparation.

The fractions enriched with either of the pigments were combined, and each pigment was purified by a CM-Sepharose column. The absorption spectrum of the eluted fraction containing the highest purity of gecko green (99.3%) or blue (76%) is shown in Figure 3. The absorption spectrum of gecko blue (curve 2 in Figure 4) was calculated by subtracting the spectrum of 24% gecko green from the spectrum of the gecko blue-enriched fraction.

An SDS−PAGE analysis of the gecko green sample (Figure 5, lane 2) showed three major bands of 37, 80, and 120 kDa which may correspond to monomer, dimer, and trimer of gecko green, respectively. Three other minor bands of 17, 30, and >130 kDa were not identified, though the 30-kDa band might be ConA, originated from ConA-Sepharose column. On the other hand, the gecko blue-enriched sample (Figure 5, lane 3) showed a 38-kDa band, which may correspond to gecko blue. The molecular masses of gecko green (37 kDa) and blue (38 kDa) thus estimated were slightly smaller than those calculated from their amino acid sequences (40 735 and 39 686, respectively; Kojima et al., 1992).

Photoreaction of Gecko Green at Liquid Nitrogen Temperature. Figure 6A,B shows typical photoreactions of gecko green at −190 °C. When the gecko green sample (curve 1 in Figure 6A) was irradiated with a green light (501 nm) at

[2] As a measure of the amount of a visual pigment in the sample, we use the unit ΔOD·mL, which is a product of a volume (mL) of the sample and a maximal optical density (ΔOD) of the difference spectrum between the pigment and its photoproduct (retinal oxime plus opsin).

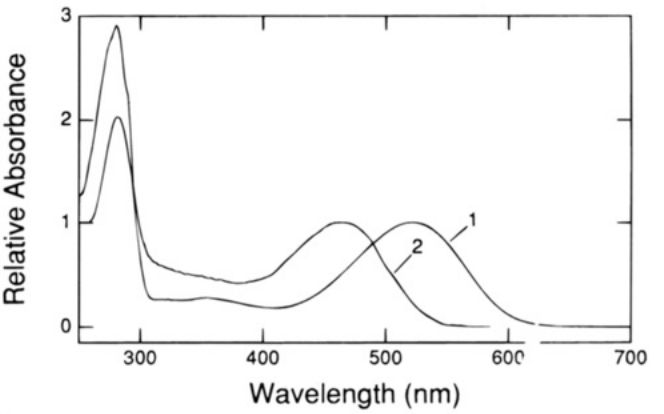

FIGURE 4: Absorption spectra of gecko green and blue in the CHAPS-PC system. The absorption spectra of green (curve 1) and blue (curve 2) were obtained from the spectra of the fraction eluted from the CM-Sepharose column. A detailed procedure for calculations is described in the text. The absorbance at the maximum of each spectrum was normalized to be 1.0. The spectrum of gecko blue was smoothed (curve 2).

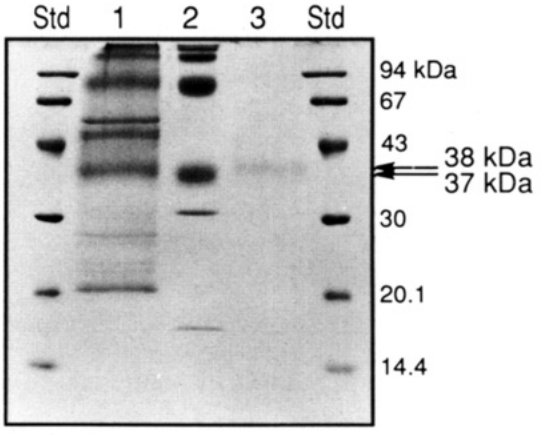

FIGURE 5: SDS−PAGE analysis of fractions containing gecko green and blue. The SDS−PAGE was performed according to the method of Laemmli (1970) using 13% polyacrylamide gel. The sample loaded in lane 1 was an aliquot of CHAPS-PC extract of gecko retinas and contained 9.6×10^{-3} ΔOD·mL of green and 9.6×10^{-4} ΔOD·mL of blue.[2] The sample loaded in lane 2 was an aliquot of an eluate from CM-Sepharose column chromatography of gecko green and contained 9.6×10^{-3} ΔOD·mL of green. The sample loaded in lane 3 was an aliquot of an eluate from CM-Sepharose column chromatography of gecko blue and contained 3.0×10^{-4} ΔOD·mL of green and 9.6×10^{-4} ΔOD·mL of blue (see also text).

−190 °C, the absorption spectrum was shifted to longer wavelength (curves 2−5), indicating the formation of batho intermediate of gecko green (batho-green). Prolonged irradiation (curves 6−11) caused a decrease of absorbance at the maximum probably due to the formation of 9-*cis* pigment, and finally produced a photosteady-state mixture of gecko green, batho-green, and the 9-*cis* pigment. The formation of the photosteady-state mixture was confirmed by the photoreversibility among these three pigments (Figure 6B). These photoreactions among the three isomers were similar to those observed in the case of rhodopsin (Yoshizawa & Wald, 1963) or iodopsin (Yoshizawa & Wald, 1967; Imamoto et al., 1989). Thus, gecko green converts to its batho intermediate, probably through a *cis*−*trans* isomerization of the chromophore like rhodopsin and iodopsin.

Thermal Reaction of Batho Intermediate of Gecko Green. To follow the thermal reactions of batho-green, the photosteady-state mixture formed at −190 °C was warmed in a stepwise manner to −110 °C in the dark (Figure 6C). Up

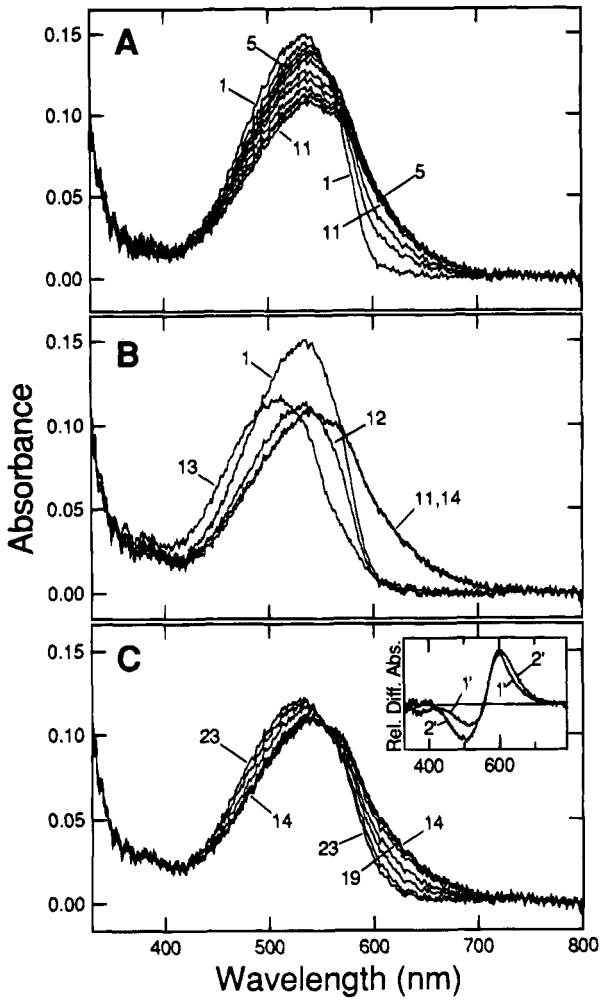

FIGURE 6: Photochemical reaction of gecko green at liquid nitrogen temperature and thermal reaction of its batho intermediate. (Panel A) Purified gecko green in 71% (v/v) glycerol was cooled to −190 °C (curve 1) and irradiated with a green light (501 nm) for a total of 5, 10, 20, 40, 80, 160, 320, 640, 1280, and 2560 s (curves 2−11, respectively). (Panel B) The original gecko green (curve 1) was irradiated with the green light (501 nm) for 2560 s to form a photosteady-state mixture (curve 11). It was then irradiated with a deep-red light (>670 nm) for 320 s (curve 12), followed by irradiation with an orange light (>560 nm) for 384 min (curve 13). The sample was irradiated again with the green light (501 nm) for 320 s (curve 14). (Panel C) The photosteady-state mixture containing mainly batho-green (curve 14) was warmed in the dark in a stepwise manner to −180, −170, −160, −150, −140, −130, −120, −110, and −100 °C (curves 15−23, respectively). All the spectra were measured at −190 °C after the sample was warmed to the required temperature. (Inset) Difference spectra between gecko green and batho-green (curve 1′), which was calculated by subtracting curve 1 from curve 2 in panel A, and between curves 14 and 23 in panel C (curve 2′), which were normalized at the λ_{max}.

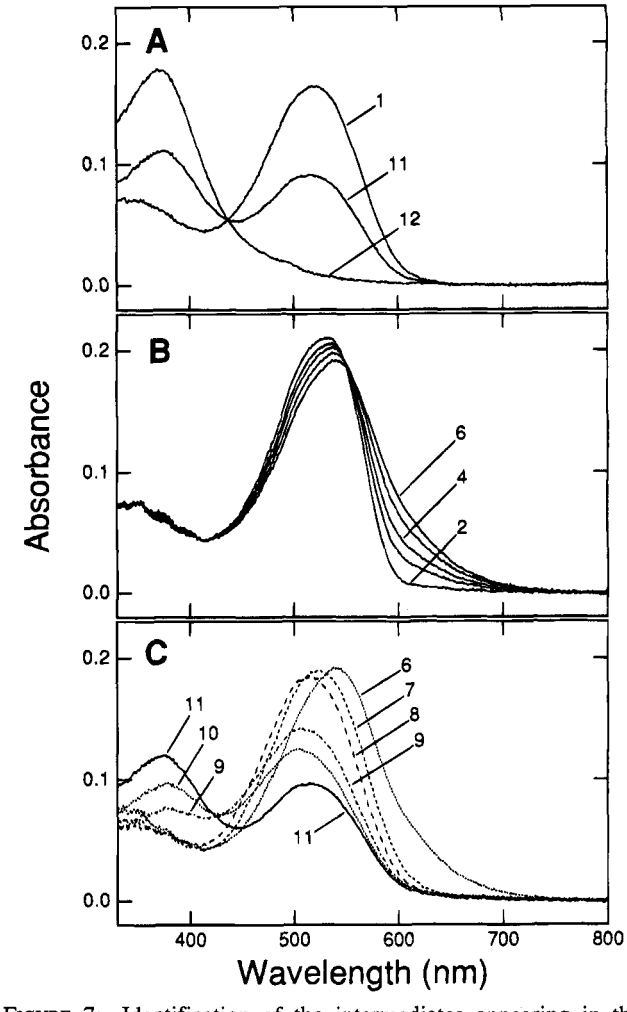

FIGURE 7: Identification of the intermediates appearing in the photobleaching process of gecko green. Purified gecko green in 71% (v/v) glycerol (curve 1, measured at 0 °C) was cooled to −196 °C (curve 2) and irradiated with a green light (501 nm) for a total of 5, 10, 20, and 40 s (curves 3−6, respectively). The irradiated sample at −196 °C (curve 6) was continuously warmed to 20 °C with spectral measurements at −80 °C (curve 7), −40 °C (curve 8), −20 °C (curve 9), and 0 °C (curve 10). The sample was further warmed to 20 °C and incubated until no spectral change was observed, followed by a spectral measurement at 0 °C (curve 11). Finally, the sample was irradiated extensively with a yellow light (>500 nm) at 20 °C, and the spectrum was measured at 0 °C (curve 12).

to −160 °C, no spectral change was observed (curves 14−18 in Figure 6C), indicating that the batho-green was stable below −160 °C. Above −150 °C, the absorption spectrum was blue-shifted with concurrent increase of the absorbance (curves 19−23 in Figure 6C). In the inset of Figure 6C, the difference spectrum calculated from curves 14 and 23 (curve 2′) was compared with the difference spectrum (curve 1′) between batho-green and the original gecko green. The disagreement of the spectral shape of the two spectra showed that batho-green did not thermally revert to the original gecko green but converted to a subsequent photobleaching intermediate. This was further confirmed by the fact that warming of the sample to 20 °C resulted in dissociation of *all-trans*-retinal from the protein moiety (data not shown). The ratio of bleached/original green (percentage of bleaching) in the experiment was estimated to be 34.0%, which was much higher than that estimated from the similar experiments on iodopsin (7.6%; Imamoto et al., 1989). It should be noted, however, that the percentage of bleaching observed in the experiments of gecko green is much lower than that observed in rhodopsin [58% (Horiuchi et al., 1980)]. This could be due to a relative abundance of 9-*cis* pigment of gecko green in the photosteady-state mixture formed at low temperature. In fact, short-time irradiation of gecko green at −196 °C caused formation of only batho-green and resulted in bleaching of a larger amount of gecko green (46.6%) when warmed to 20 °C (Figure 7A, see below).

Intermediates in the Bleaching Process of Gecko Green. To estimate the number of intermediates existing in the

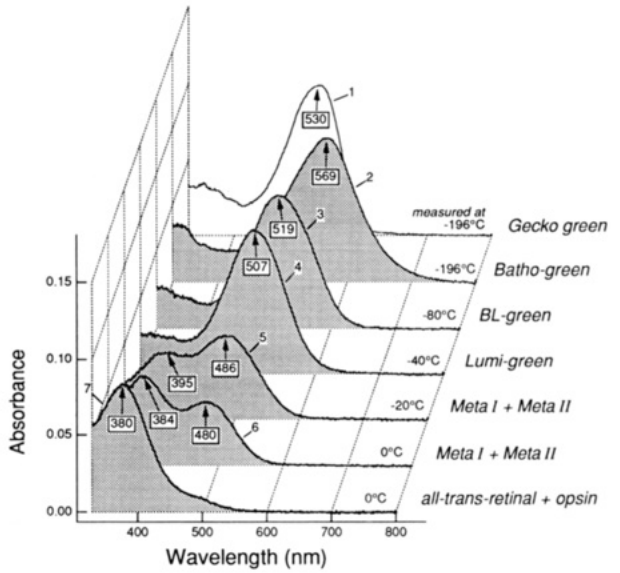

FIGURE 8: Calculated absorption spectra of the intermediates in the photobleaching process of gecko green. Curves 1, 2, 3, 4, 5, 6, and 7 are the absorption spectra calculated by subtracting the spectra of unbleached gecko green (52.7%) measured at the corresponding temperature from curves 2, 6, 7, 8, 9, 10, and 11 in Figure 7, respectively. Boxed values are the absorption maxima (nm).

photobleaching process of gecko green, we first irradiated a gecko green sample at −196 °C with the green light for 40 s to avoid the formation of 9-*cis* pigment and then warmed the sample to follow the spectral changes. As shown in Figure 7B, irradiation of the gecko green sample for only 40 s resulted in red-shift of the spectrum with an isosbestic point at 550 nm, suggesting that only batho-green was produced by the irradiation. In the subsequent warming of the irradiated sample (Figure 7C), we are able to identify five intermediates in the bleaching process of gecko green. The first intermediate detected by this experiment is batho-green (curve 6), which was stable up to −160 °C. The following two intermediates were stable up to −80 °C (curve 7) and −40 °C (curve 8), respectively. At −20 °C (curve 9) and 0 °C (curve 10), a mixture of at least two additional intermediates was observed. Finally, *all-trans*-retinal (λ_{max} = 380 nm, curve 11 in Figure 7) was dissociated from the protein moiety. The sample after warming to 20 °C (curve 11 in Figure 7C) contained 52.7% gecko green, 46.6% *all-trans*-retinal plus opsin, and 0.7% 9-*cis* pigment (Figure 7A). These values were estimated by fitting the spectral shape of curve 11 in Figure 7 with the spectrum of a combination of gecko green, 9-*cis* pigment, and *all-trans*-retinal. The absorption spectrum of the 9-*cis* pigment was calculated by the method reported for estimation of the spectrum of isorhodopsin (Yoshizawa & Shichida, 1982).

Since the amount of 9-*cis* pigment contained in the sample was negligible, we were able to calculate the spectra of several intermediates by subtracting the spectrum of residual gecko green from curves 6−11 in Figure 7C. As shown in Figure 8, the calculation gave us absorption spectra of batho-green (curve 2), BL-green (curve 3), and lumi-green (curve 4), displaying absorption maxima at 569, 519, and 507 nm, respectively. On the other hand, the spectra of the following intermediates, meta-green I and II, were not estimated by this procedure, probably because these intermediates formed an equilibrium state mixture. Only the absorption maxima (λ_{max}) of these intermediates were estimated to be located at about 486 nm (meta-green I) and 384 nm (meta-green II), which were the λ_{max} of curve 5 and 6, respectively.

Thermal Behavior of Lumi, Meta I, and Meta II Intermediates of Gecko Green. To make clear the thermal reactions of the later intermediates, lumi, meta I, and meta II, we measured the spectral changes of gecko green at a constant temperature (−40, −20, or −10 °C) after the irradiation. Irradiation of gecko green at −40 °C converted it to mainly lumi-green (curve 1 in Figure 9A), but a small positive absorbance at about 610 nm indicates a little formation of BL-green. In fact, the subsequent incubation resulted in decrease of absorbance near the wavelengths (curves 1′ and 2′ in Figure 9A′), and similar spectral changes were observed in the formation of BL-green and its conversion into lumi-green at −80 °C (data not shown). In the course of following incubation at −40 °C, we observed at least two additional phases of the spectral changes. The second phase (curves 3′−5′ in Figure 9A′) is the decrease of absorbance at about 530 nm with a concurrent increase at about 410 nm, and the third phase (curves 6′−9′ in Figure 9A′) is the decrease of absorbance at about 520 nm with an increase at about 395 nm. These two phases reflect mainly the conversion process of lumi-green to meta-green I and that of meta-green I to II, respectively. However, a similarity in the spectral changes between the second and third phases at the longer wavelengths could be explained only by the presence of thermal back-reactions of meta-green I and meta-green II to the respective precursors, lumi-green and meta-green I (see Discussion).

In the experiment at −20 °C (Figure 9B), the spectrum measured immediately after the irradiation showed a shoulder at about 470 nm and a peak at about 410 nm (curve 1 in Figure 9B), indicating the conversion of gecko green to a mixture of meta-green I and II. Spectral changes during the subsequent incubation were separated into three phases. The first phase (curves 1′ and 2′ in Figure 9B′) is the decrease of absorbance at about 520 nm and the increase at about 390 nm, which is similar in spectral shape to the third phase observed at −40 °C (curves 6′−9′ in Figure 9A′). In the second phase, we observed a decrease of absorbance at a broad region of wavelengths from 410 to 600 nm (curves 3′−6′ in Figure 9B′). Although this change could be explained by the concurrent decrease of lumi-green and meta-green I, the spectral shape was not simulated by the spectra of only these intermediates (data not shown), suggesting a formation (or decay) of another intermediate during the incubation. The most plausible explanation for this is a formation of meta-green III whose absorption maximum is located at about 500 nm (curves 7′−9′ in Figure 9B′) because the subsequent spectral change (the third phase) clearly showed the decrease of absorbance at about 500 nm. The spectral change observed in the third phase is probably due to the conversion of meta-green III to *all-trans*-retinal and opsin.

At −10 °C, we observed spectral changes composed of only two phases (Figure 9C′) which were similar to the second and third phases of spectral changes observed at −20 °C, respectively.

Meta Intermediates of Gecko Blue. To follow the photobleaching process of gecko blue, we employed similar low temperature time-resolved spectroscopy and identified three meta intermediates of gecko blue. Since the gecko blue sample used for the experiments contained some amount of

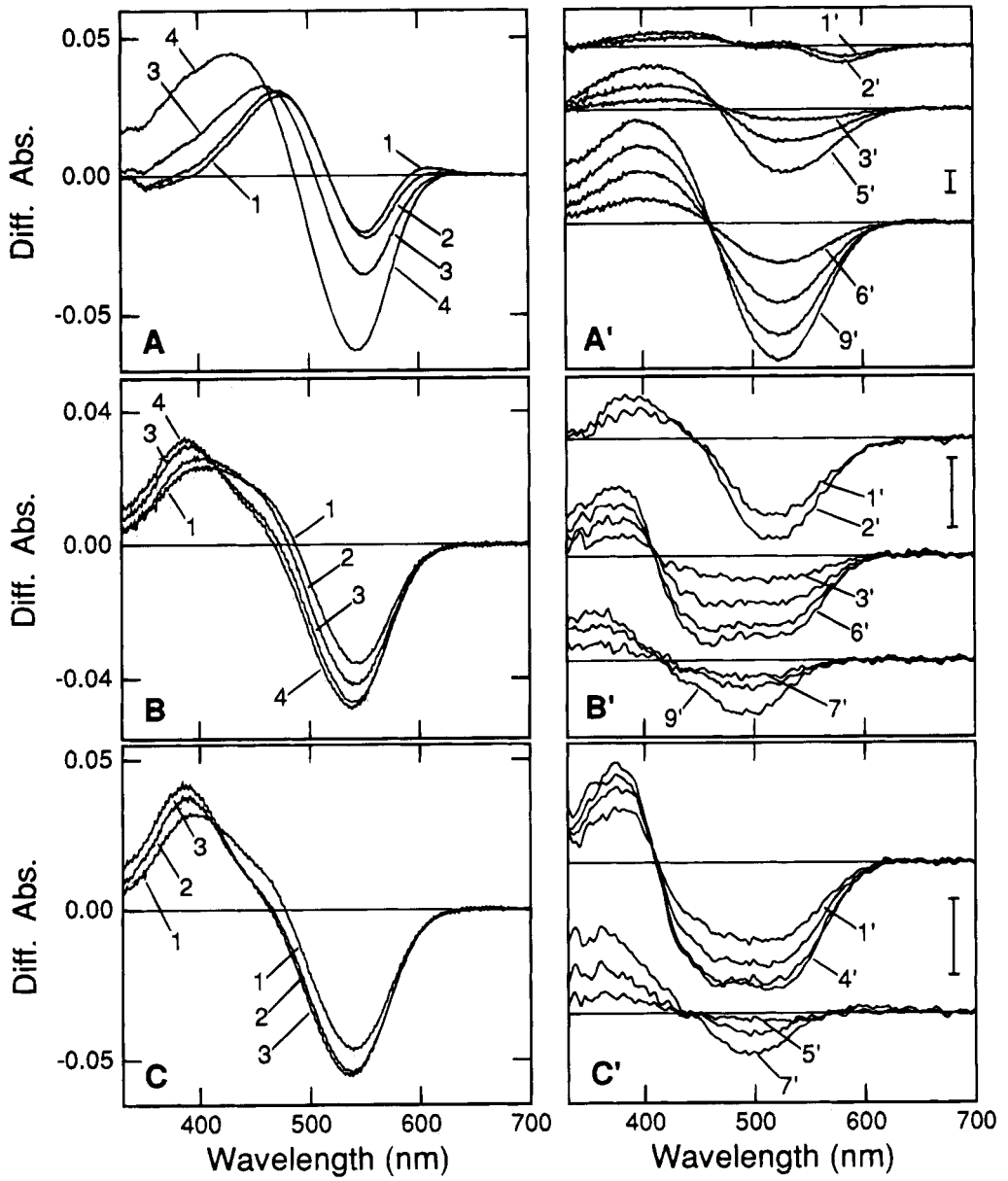

FIGURE 9: Thermal reactions of intermediates observed after irradiation of gecko green at low temperatures. Purified gecko green in 71%-(v/v) glycerol was cooled to −40 °C (A), −20 °C (B), or −10 °C (C) and irradiated with a red light (>580 nm) for 30 s. Then it was incubated at the same temperature for recording the spectra. (Panel A) Difference spectra between the spectrum recorded before irradiation and those recorded immediately (0 min, curve 1), 5 min (curve 2), 40 min (curve 3), and 640 min (curve 4) after irradiation at −40 °C. (Panel B) Difference spectra obtained at −20 °C. The time after irradiation is 0 min (curve 1), 5 min (curve 2), 80 min (curve 3), or 640 min (curve 4). (Panel C) Difference spectra obtained at −10 °C. The time after irradiation is 0 min (curve 1), 20 min (curve 2), or 160 min (curve 3). (Panel A′) Curves 1′ and 2′ are the difference spectra between curve 1 in A and the spectra recorded at 2.5 and 5 min after irradiation. Curves 3′−5′ are those between curve 2 in A and the spectra at 10, 20, and 40 min after irradiation. Curves 6′−9′ are those between curve 3 in A and the spectra at 80, 160, 320, and 640 min after irradiation. (Panel B′) Curves 1′ and 2′ are the difference spectra between curve 1 in B and the spectra recorded at 2.5 and 5 min after irradiation. Curves 3′−6′ are those between curve 2 in B and the spectra at 10, 20, 40, and 80 min after irradiation. Curves 7′−9′ are those between curve 3 in B and the spectra at 160, 320, and 640 min after irradiation. (Panel C′) Curves 1′−4′ are the difference spectra between curve 1 in C and the spectra recorded at 2.5, 5, 10, and 20 min after irradiation. Curves 5′−7′ are those between curve 2 in C and the spectra at 40, 80, and 160 min after the irradiation. Scale bars in panels A′−C′ indicate 5 × 10^{-3} of absorbance unit.

gecko green, we first irradiated the sample at 0 °C with a red light (>610 nm) to bleach only gecko green contained in the sample. Then the sample was subjected to low temperature time-resolved spectroscopy at −20 or −10 °C.

Figure 10 panels A and A′ show the spectral changes observed at −20 °C. Irradiation of gecko blue at −20 °C resulted in an increase of absorbance at the wavelength region from 340 to 490 nm, displaying a maximum at about 450 nm and a shoulder at about 380 nm (curve 1 in Figure 10A). This spectral change was ascribed to the formation of a mixture of meta-blue I and II. Three phases of spectral changes were observed during the subsequent incubation at this temperature. At the first stage, a decrease of absorbance at about 470 nm and an increase at about 370 nm were observed (curves 1′ and 2′ in Figure 10A′). Second, a small increase of absorbance at about 530 nm, a decrease at 460 nm, and an increase at 370 nm (curves 3′ and 4′ in Figure 10A′) were concurrently observed. Finally, a large decrease of absorbance at about 475 nm and an increase at about 380 nm were observed. The initial spectral change in the thermal

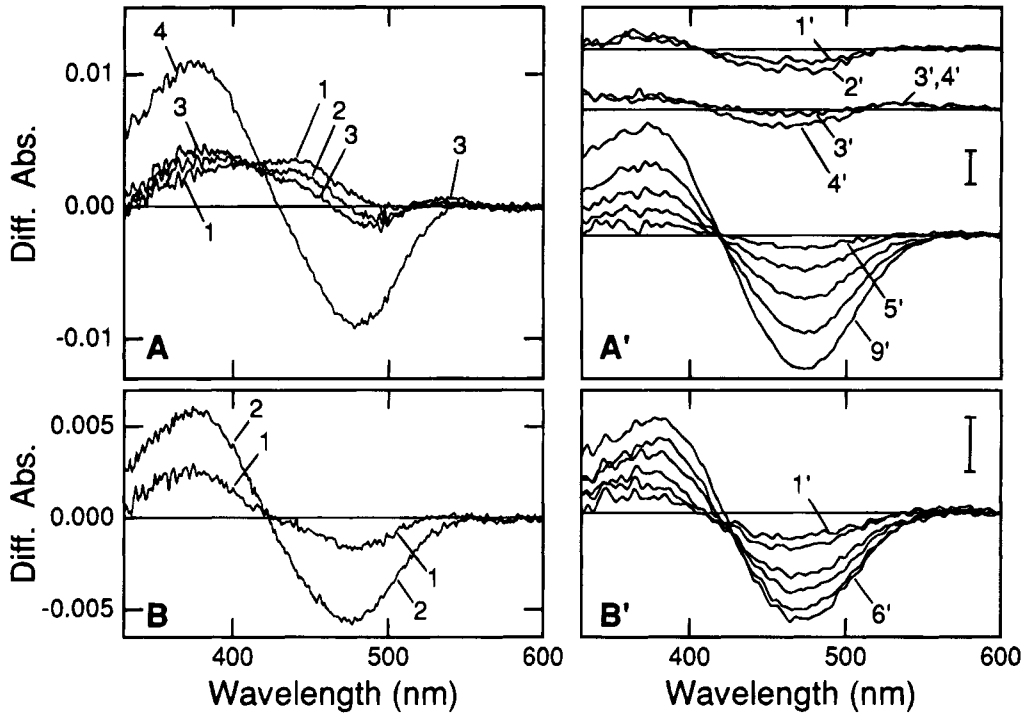

FIGURE 10: Thermal reaction of meta intermediates of gecko blue. A mixture of gecko blue (76%) and green (24%) in 71%(v/v) glycerol was irradiated with a red light (>610 nm) at 0 °C until gecko green completely bleached. It was then cooled to −20 °C (A) or −10 °C (B) and irradiated with an orange light [>520 nm (A) or >530 nm (B)] for 30 s. The spectra were recorded at adequate times after the irradiation. (Panel A) Difference spectra between the spectrum before irradiation and those recorded immediately (0 min, curve 1), 5 min (curve 2), 20 min (curve 3), and 640 min (curve 4) after irradiation at −20 °C. (Panel B) Difference spectra obtained at −10 °C. The time after irradiation is 0 min (curve 1) and 80 min (curve 2) after irradiation. (Panel A′) Curves 1′ and 2′ are the difference spectra between curve 1 in A and the spectra recorded at 2.5 and 5 min after irradiation. Curves 3′ and 4′ are those between curve 2 in A and the spectra at 10 and 20 min after irradiation. Curves 5′−9′ are those between curve 3 in A and the spectra at 40, 80, 160, 320, and 640 min after irradiation. (Panel B′) Difference spectra between curve 1 in B and the spectra recorded at 2, 4, 10, 20, 40, and 80 min after irradiation (curves 1′−6′). Scale bars in panels A′ and B′ indicate 2 × 10^{-3} of absorbance unit.

reaction could be due to the conversion of meta-blue I to II, and the final change was ascribed to a conversion of meta-blue III to *all-trans*-retinal and opsin. Then the second spectral change seemed to reflect a combined conversion of meta-blue I to II and of meta-blue II to III.

When irradiated at −10 °C, gecko blue converted mainly to meta-blue III with a small contamination of meta-blue II and/or *all-trans*-retinal dissociated from opsin (curve 1 in Figure 10B). During the subsequent incubation, meta-blue III converted to *all-trans*-retinal and opsin (curves 1′−6′ in Figure 10B′), and the spectral change was almost identical with the final spectral change observed at −20 °C (curves 5′−9′ in Figure 10A′).

Kinetics of Meta II Intermediates of Gecko Green and Blue. In order to estimate the apparent time constants for formation and decay of meta II intermediates, the absorbance changes at 380 nm after irradiation of gecko green and blue at −20 °C were measured (Figure 11A,B). These data were fitted by three sequential single-exponential curves, whose time constants were 0.9, 12, and 180 min for gecko green or 6.3, 36, and 210 min for gecko blue (Table 1). Each of the three time constants corresponds to that of the respective part of the triphasic spectral changes as shown in Figure 9B′ (gecko green) or in Figure 10A′ (gecko blue). The fastest (0.9 min for gecko green or 6.3 min for gecko blue) of the time constants reflects the formation process of only the meta II intermediate, the second (12 min for gecko green or 36 min for gecko blue) corresponds to both the formation and decay processes of meta II intermediate, and the third (180 min for gecko green or 210 min for gecko blue) is due to the decay process of meta III intermediate to *all-trans*-retinal and opsin.

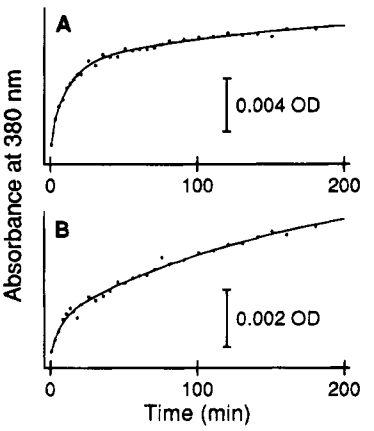

FIGURE 11: Kinetic profiles of gecko meta-green II and meta-blue II. Absorbance changes at 380 nm after irradiation of gecko green (panel A) and blue (panel B) were measured at −20 °C. The data in panels A and B were recorded in the experiments as shown in panel B of Figure 9 (green) and in panel A of Figure 10 (blue), respectively. The smooth curve in each panel is a combination of three sequential single-exponential curves with time constants shown in Table 1.

For comparison, the time courses of formation and decay of meta II intermediate of chicken rhodopsin (metarhodopsin II) were also measured (data not shown). The absorbance change at 380 nm after irradiation of chicken rhodopsin at −20 °C was fitted with three sequential single-exponential

Table 1: Formation and Decay Time Constants of Meta II and Meta III Intermediates at −20 °C

	meta II formation[a]	meta II to III conversion[a]	meta III decay[a]
gecko green	0.9	12	180
gecko blue	6.3	36	210
chicken rhodopsin	6.7, 68	3100	ND[b]

[a] Time constants calculated from the data in Figure 11 (minutes). [b] Not determined.

curves having time constants of 6.7, 68, and 3100 min (Table 1). Among these three phases, the first and second ones (time constants of 6.7 and 68 min) showed increases of absorbance. Both the phases are due to the formation of metarhodopsin II (see Discussion). In the third phase (a time constant of 3100 min), a decrease of absorbance was observed, indicating the decay of metarhodopsin II into metarhodopsin III.

DISCUSSION

In the present study, we have detected several photobleaching intermediates of gecko visual pigments by low temperature spectroscopy using the purified samples from the retinas by two steps of column chromatography. The photobleaching processes are thus summarized as shown in Figure 12. It should be noted that gecko green, as well as gecko blue, has a meta III intermediate in its bleaching process, although earlier reports failed to detect this intermediate (Bowmaker, 1973; Crescitelli, 1977b). In addition, we have detected a BL intermediate of gecko green, whose spectral character is similar to that of iodopsin. The comparison of the absorption spectra and thermal behavior of intermediates of gecko visual pigments with those of other visual pigments reveals several interesting characteristics similar or different among visual pigments.

Absorption Spectra of Gecko Green and Blue in CHAPS-PC System. It has been reported that absorption maxima of gecko green and blue solubilized in 2% digitonin are at 521 and 478 nm, respectively (Crescitelli, 1963). While the former value is identical with that estimated in the photoreceptor cells by the microspectrophotometry, the latter is significantly diverged from that (467 nm) in the cells (Crescitelli et al., 1977; Loew, 1994). The disagreement of absorption maximum of gecko blue might be due to the difference in environment between the digitonin-solubilized gecko blue and that in photoreceptor cells. The value estimated in the present study (465 nm) is in good agreement with that reported by the microspectrophotometry (Crescitelli et al., 1977; Loew, 1994), suggesting that the native conformation of gecko blue is preserved in CHAPS-PC system better than in digitonin.

Thermal Behavior of Batho-Green. Since gecko green has not only an amino acid sequence quite similar (82.6% identical) to that of iodopsin but also a chloride binding site which is one of the common properties of group L visual pigments including iodopsin, we predicted that thermal behavior of batho-green would be similar to that of iodopsin. However, the present results clearly show that, unlike bathoiodopsin, batho-green does not thermally revert to the original gecko green but converts to the next intermediate. Therefore, the thermal reversion of batho intermediate at low temperature is not a common property of the group L visual

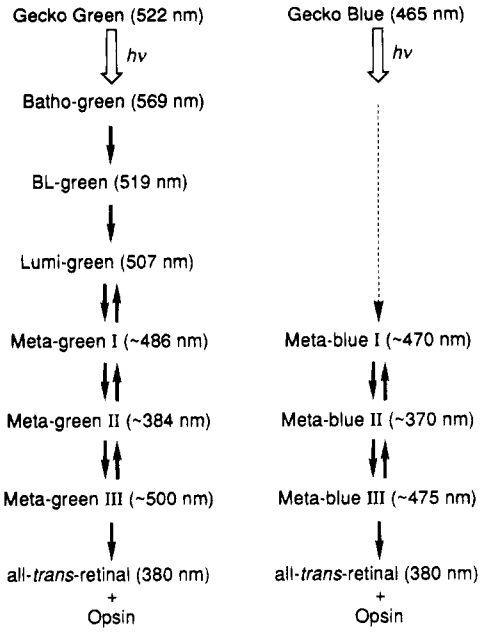

FIGURE 12: Photobleaching processes of gecko green (left) and blue (right). The absorption maximum (nm) of each intermediate is represented in parentheses.

pigments. Since the batho intermediate of either rhodopsin or chicken green (Imai et al., manuscript in preparation) displays no reversal reaction, the reversal reaction is unique for iodopsin. It is therefore of interest to speculate which amino acid residues induce the reversal reaction in iodopsin.

Since the batho intermediate of rhodopsin decays to the next intermediate with changes in chromophore−opsin interaction near the cyclohexenyl ring of the chromophore (Okada et al., 1991) and the cyclohexenyl ring locates near helices 3 and 6 (Nakayama & Khorana, 1990), the residues that induce the reversal reaction in iodopsin should be located in helices 3 and 6. Comparisons of amino acid residues of iodopsin with those of gecko green, chicken green, and rhodopsins enable us to select two phenylalanines at the positions 276 and 283 in iodopsin as the putative amino acid residues. At each position, the other three visual pigments have an amino acid residue having a smaller side chain (I, L, V, G, or S). This speculation will be confirmed by the low temperature experiments of iodopsin mutants, in which each of these phenylalanine residues is substituted by a residue having a smaller side chain.

Presence of BL Intermediate in the Bleaching Process of Gecko Green. Our results show the presence of a BL intermediate in the bleaching process of gecko green. Like BL intermediate of iodopsin (Shichida et al., 1993), BL-green displayed its absorption maximum just between batho- and lumi-green, and its extinction coefficient was comparable to those of batho- and lumi-green. These characteristics are different from those of BL(BSI) intermediate of rhodopsin (Shichida et al., 1981; Hug et al., 1990), which displays an absorption maximum at shorter wavelength and an extinction coefficient much smaller than those of both batho- and lumirhodopsins. Furthermore, low temperature spectroscopy hardly detected a putative BL intermediate in the photobleaching process of chicken green (Imai et al., manuscript in preparation), which is consistent with the result of rhodopsin (Yoshizawa & Shichida, 1982; Hug et al., 1990), while BL intermediate of iodopsin was easily detected by

low temperature spectroscopy (Imamoto et al., manuscript in preparation). Thus we can speculate that, in addition to the location of absorption maximum and the magnitude of extinction coefficient, easy detection of BL intermediate at low temperature is unique for a group L visual pigment which has a chloride binding site in its protein moiety.

Thermal Equilibrium between Lumi and Meta I Intermediates of Gecko Visual Pigments. In the warming experiment of batho-green from −190 °C (Figure 7C), we observed stepwise changes in spectrum due to batho-to-BL and BL-to-lumi transitions, but the spectral changes due to lumi-to-meta I transition were not able to be separated from those due to meta I-to-meta II transition. Although low temperature time-resolved spectroscopy enabled us to identify the spectral changes due to the decays of lumi and meta I intermediates, the separations of the respective reaction processes are not complete. These results might be explained by either of the following two reasons: one is that decay time constant of lumi-green is comparable to the formation and decay time constants of meta-green I and meta-green II, and the other is the presence of thermal equilibria among lumi, meta I, and meta II intermediates of gecko green. A noteworthy point is that, at −20 or −10 °C, the decay of lumi-green was observed not only in the process of formation of meta-green I but also in those of meta-green II and III (Figure 9). If the thermal equilibria among these intermediates is absent, these results should be accounted for by the comparable decay time constants between lumi-green and meta-green II. However, the decay process of lumi-green at −40 °C was accompanied by the formation of meta-green II but not by its decay (Figure 9), indicating that comparable decay time constants between these intermediates are unlikely. Therefore, our results highly suggest the presence of thermal equilibria among lumi, meta I, and meta II intermediates of gecko green, by which the decay of lumi-green is observed even in the formation process of meta-green III. Recently, we confirmed the presence of thermal equilibria among lumi, meta I, and meta II intermediates of rhodopsin by direct measurements of thermal back reactions from metarhodopsin I to lumirhodopsin and metarhodopsin II to metarhodopsin I (Imai et al., 1994). Since the presence of thermal equilibria among these intermediates is also suggested in iodopsin (Shichida et al., 1993) or chicken green (Imai et al., manuscript in preparation), we can safely conclude that the thermal equilibria among these intermediates are common in all the visual pigments.

Thermal Behavior of Meta Intermediates of Gecko Visual Pigments. Time-resolved low temperature spectroscopy enabled us to estimate the apparent time constants for the formation and decay of meta intermediates of gecko visual pigments at −20 °C (Table 1). The formation time constant of meta-green II is about 7-fold shorter than that of meta-blue II, and the time constant for the conversion from meta-green II to III is about 3-fold shorter than that from meta-blue II to III. On the other hand, the decay time constant of meta-green III is comparable with that of meta-blue III. These results indicate that meta-green II forms at an earlier time than meta-blue II, while meta-green II persists during the time comparable with meta-blue II.

In comparison between gecko visual pigments and rhodopsin systems, the most prominent difference is that the decay time constants of meta III intermediates of both the gecko visual pigments are shorter than even the formation time constant of metarhodopsin III (Table 1). The results clearly show that meta II intermediates of gecko visual pigments are less stable than metarhodopsin II. It is consistent with the idea that gecko visual pigments are cone-type ones (Kojima et al., 1992), since the meta II intermediate of chicken cone pigment, iodopsin (Shichida et al., 1993; Okada et al., 1994) or chicken green (Shichida et al., 1994), is less stable as well.

Under our experimental conditions, the formation process of metarhodopsin II was simulated by two exponential curves with time constants of 6.7 and 68 min (Table 1). Although there are several possibilities to explain the biphasic formation of metarhodopsin II (Straume et al., 1990; Thorgeirsson et al., 1993), we prefer to explain it as follows: Because our recent investigation clearly showed the presence of thermal equilibrium between lumirhodopsin and metarhodopsin I (Imai et al., 1994), the shorter time constant could reflect the formation process of metarhodopsin II before establishment of a quasiequilibrium state between lumirhodopsin and metarhodopsin I, and the longer time constant could reflect the process after establishment of the quasiequilibrium state. Therefore, the metarhodopsin II emerged with the time constant of 6.7 min at −20 °C, which is comparable to the time constant for meta-blue II formation and slower than meta-green II formation.

As described above, the present result (Table 1) showed that meta-green II forms faster than metarhodopsin II at −20 °C. However, Liang et al. (1993) reported that meta-green II formation at room temperature is slower than metarhodopsin II formation. One of the possible explanations for the apparent disagreement is that the temperature dependence of the formation of meta-green II is somewhat different from that of metarhodopsin II. That is, the activation enthalpy of the formation of meta-green II is smaller than that of metarhodopsin II. Another possibility is the difference in environment of the solubilized gecko green between these experiments. We solubilized gecko green and rhodopsin using a mixture of CHAPS and PC and purified by column chromatography, while Liang et al. (1993) solubilized these visual pigments by digitonin. Since it is generally accepted that thermal behavior of intermediates of rhodopsin is highly affected by the environment, it is essential to make the experimental conditions uniform for the comparison of the rate constants of intermediates.

In the present study, we assume that the apparent time constants reflect the thermal behavior of meta intermediates of gecko visual pigments. However, our experiments highly suggested the presence of thermal equilibria among the later intermediates of gecko visual pigments including lumi intermediates. Therefore, more detailed analysis for the reaction kinetics will give us the better understanding of the thermal behavior of meta intermediates.

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