Photochemical and Biochemical Properties of Chicken Blue-Sensitive Cone Visual Pigment†

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ABSTRACT: Through low-temperature spectroscopy and G-protein (transducin) activating experiments, we have investigated molecular properties of chicken blue, the cone visual pigment present in chicken blue-sensitive cones, and compared them with those of the other cone visual pigments, chicken green and chicken red (iodopsin), and rod visual pigment rhodopsin. Irradiation of chicken blue at −196 °C results in formation of a batho intermediate which then converts to BL, lumi, meta I, meta II, and meta III intermediates with the transition temperatures of −160, −110, −40, −20, and −10 °C. Batho intermediate exhibits an unique absorption spectrum having vibrational fine structure, suggesting that the chromophore of batho intermediate is in a C_6-C_7 conformation more restricted than those of chicken blue and its isopigment. As reflected by the difference in maxima of the original pigments, the absorption maxima of batho, BL, and lumi intermediates of chicken blue are located at wavelengths considerably shorter than those of the respective intermediates of chicken green, red and rhodopsin, but the maxima of meta I, meta II, and meta III are similar to those of the other visual pigments. These facts indicate that during the lumi-to-meta I transition, retinal chromophore changes its original position relative to the amino acid residues which regulate the maxima of original pigments through electrostatic interactions. Using time-resolved low-temperature spectroscopy, the decay rates of meta II and meta III intermediates of chicken blue are estimated to be similar to those of chicken red and green, but considerably faster than those of rhodopsin. Efficiency in activating transducin by the irradiated chicken blue is greatly diminished as the time before its addition to the reaction mixture containing transducin and GTP increases, while that by irradiated rhodopsin is not. The time profile is almost identical with those observed in chicken red and green. Thus, the faster decay of enzymatically active state is common in cone visual pigments, independent of their spectral sensitivity.

The visual transduction process in photoreceptor cells begins with photon absorption by a visual pigment, which is a member of a family of G-protein coupled receptors and contains 11-*cis*-retinal as a light-absorbing chromophore (Wald, 1968; Hargrave & McDowell, 1992). In most vertebrates, different types of visual pigments are present in rod and cone photoreceptor cells, where they mediate vision under twilight and daylight conditions, respectively. Recent investigations revealed that vertebrate visual pigments are classified into four groups of cone visual pigments and a single group of rod visual pigments rhodopsins (Okano et al., 1992). The presence of multiple types of cone visual pigments with different absorption maxima is the molecular basis of color discrimination [for recent review, see Yoshizawa (1992) and Okano et al. (1996)], and the maximum of each visual pigment is regulated by the amino acid residue(s) situated near the retinal chromophore (Neitz et al., 1991; Okano et al., 1992; Winderickx et al., 1992; Merbs & Nathans, 1993; Asenjo et al., 1994).

In addition to their unique role in color discrimination, cone photoreceptor cells exhibit photoresponse patterns different from that of rod photoreceptor cells. Cones are less sensitive to light, display faster photoresponses, and adapt more rapidly compared to rods [for recent review, see Yau (1994) and Baylor (1996)]. Since the differences would originate from the different properties of signal transduction proteins, including visual pigments, extensive studies of the molecular properties are important for furthering our understanding of the molecular mechanism leading to the difference between rod and cone photoreceptor cells. In line with this, we investigated the molecular properties of two types of cone visual pigments, chicken red (iodopsin) and chicken green, and compared them with those of rhodopsin (Shichida et al., 1993, 1994; Okada et al., 1994; Imai et al., 1995). The results showed that these pigments exhibit faster regeneration from 11-*cis*-retinal and opsin and faster forma-

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[1] Abbreviations: cRh, chicken rhodopsin; cR, chicken red-sensitive cone visual pigment; cG, chicken green-sensitive cone visual pigment; cB, chicken blue-sensitive cone visual pigment; batho, batho intermediate; lumi, lumi intermediate; BL, an intermediate formed between batho and lumi; meta, meta intermediate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; ConA, concanavalin A; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; KIU, kallikrein inhibitor units.

tion and decay of physiologically active meta II intermediates than does the rhodopsin, as expected, due to the functional difference between rod and cone photoreceptor cells. On the other hand, photochemical and subsequent thermal reactions at an early stage in chicken green are almost identical with those in rhodopsin but different from those in chicken red (Shichida et al., 1993; Imai et al., 1995; Imamoto et al., 1996). Since the differences correlate well with those in amino acid sequence and absorption maximum among three visual pigments, amino acid residues including color-regulating ones might affect the early bleaching process of visual pigments. Thus, it is of interest to investigate the relationship between color of original visual pigment and early bleaching process and to confirm that the faster decay of meta II intermediate is a common property of cone visual pigments.

In the present study, we have investigated the photobleaching process of chicken blue, the cone visual pigment present in chicken blue-sensitive cones, and its efficiency in activating transducin. Chicken blue belongs to a family of one of the middle-wavelength-sensitive visual pigments (middle1) among the four families (short, middle1, middle2, and long) of visual pigments (Yoshizawa, 1992; Okano et al., 1992, 1996). While the photobleaching processes of some cone-type visual pigments belonging to long (chicken red, gecko green), middle2 (chicken green, gecko blue), and short (*Xenopus* violet) families are reported previously (Yoshizawa and Wald, 1967; Hubbard & Kropf, 1959; Imamoto et al., 1989; 1994; 1996; Shichida et al., 1993; 1994; Okada et al., 1994; Lewis et al., 1995; Kojima et al., 1995; Imai et al., 1995; Tachibanaki et al., 1995; Starace and Knox, 1997), there were no reports on those belonging to middle1 family. In addition to its unique amino acid sequence, chicken blue exhibits a unique absorption spectrum whose maximum (455 nm) is similar to that of protonated Schiff's base (about 440 nm) in organic solution, suggesting that there are only a few amino acid residues which regulate the maximum of chicken blue. Current findings show that chicken blue converts to a unique batho intermediate whose absorption spectrum exhibits vibrational fine structure. The batho intermediate converts to a BL intermediate like that of chicken red, and then decays to all-*trans*-retinal and opsin through lumi, meta I, meta II, and meta III intermediates. On the other hand, like meta II of chicken red and chicken green, meta II of chicken blue exhibits faster decay than that of rhodopsin. Furthermore, chicken blue exhibits a time-dependent profile in activating transducin similar to those of chicken red and chicken green but considerably different from that of rhodopsin. The molecular properties of chicken blue are discussed on the basis of amino acid residues which regulate the maxima of the visual pigments and those which regulate the thermal reactions of the visual pigments.

MATERIALS AND METHODS

Sample Preparation. Chicken blue was extracted from fresh chicken retinas by a mixture of CHAPS and PC and purified by means of affinity and ion-exchange column chromatographies. The fraction containing mainly chicken blue was eluted from concanavalin A-Sepharose affinity column (Pharmacia) as reported previously (Okano et al., 1989; Imai et al., 1995). For further purification, the fraction was applied to a SP-Sepharose ion-exchange column (Pharmacia) which had been equilibrated with buffer A [20% glycerol (w/v), 0.6% CHAPS, 0.8 mg/mL PC, 50 mM HEPES, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU aprotinin, pH 6.6], and chicken blue was eluted with a linear gradient of NaCl (100 to 500 mM) in buffer A. After lowering the NaCl concentration of the chicken blue fraction to 10 mM, chicken red, green, and rhodopsin contaminating in the fraction were bleached by irradiation with >590 nm light for 40 min at 0 °C in the presence of 10 mM hydroxylamine. Retinal-oxime produced by the irradiation was removed from the fraction using CM-Sepharose column (Pharmacia), followed by concentration with ultra filtration membrane (AMICON YM-30). For low-temperature spectroscopy, an equal volume of glycerol was added to the sample to freeze the sample transparently. For GTPase assay, the purified pigments were incorporated into PC liposome by dialysis at 4 °C for 18 h with five buffer changes against 300-fold volumes of buffer B [50 mM HEPES, 140 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 1 μg/mL aprotinin, and 1 μg/mL leupeptin, pH 6.5 at 4 °C]. Transducin was prepared from the fresh bovine retina by the method previously reported (Fukada et al., 1989a).

Spectrophotometry. The system for the measurements of absorption spectra was reported previously (Imai et al., 1995). Absorption spectra were recorded with a Shimadzu Model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. Oxford model CF-1204 cryostat was used for low-temperature spectroscopy. The sample temperature was regulated to within 0.1 °C by a temperature controller (ITC-4, Oxford) attached to the cryostat. The sample was irradiated with light from a 1 kW tungsten halogen lamp (Rikagaku Seiki). The wavelength of the irradiation light was selected with a glass cutoff filter (VY50, VO54, 59, VR61, 66; Toshiba), or an interference filter (436 nm; Nihonshinku). In time-resolved, low-temperature spectroscopy, the amount of visual pigment in the sample bleached by the 30 s irradiation (>520 nm) was about 50% which was estimated by complete bleaching after each experiment (Imai et al., 1994).

Calculation of Absorption Spectra of Intermediates and iso Pigments. Absorption spectra of intermediates were calculated by the methods described previously (Yoshizawa & Shichida, 1982; Imai et al., 1994, 1995). Briefly, the spectra of original pigment were first recorded at selected temperatures to estimate temperature-dependent changes of the spectra. Then the sample was irradiated at -196 °C to produce a photo-steady-state mixture containing mainly iso pigment. After recording the spectra of the mixture at the selected temperatures, it was bleached in a stepwise manner in the presence of 10 mM hydroxylamine at 0 °C. Since the mixture contained only original and iso pigments, changes in absorbance at the isosbestic point (440 nm) as a function of irradiation time reflected the amounts of these pigments (Yoshizawa & Wald, 1963). Therefore, the spectra of iso pigment at the selected temperatures were calculated by subtracting the spectra of the estimated amount of original pigments from the spectra of the mixture at these temperatures. The spectra of iso pigments were normalized to represent an equivalent amount of original pigment.

From the spectrum of a mixture composed of intermediate, original, and iso pigments, we calculated the spectrum of intermediate as follows. First, the amounts of residual original and iso pigment present in the mixture were estimated as reported previously (Imai et al., 1994). Then

the spectrum of intermediate was calculated by subtracting the spectra of known amounts of original and isopigments from that of the mixture and normalized to represent an equivalent amount of original pigment.

Transducin Activation Assay of Rod and Cone Visual Pigments. The amount of transducin activated by the irradiated pigments was measured as GTPase activities of transducin as reported previously (Imai et al., 1997). Namely, the pigment suspension was irradiated with orange light (cB, >520 nm; cRh and cG, >570 nm; cR, >640 nm) for 30 s at 2 °C. After the indicated preincubation time in the dark at this temperature, 20 μL aliquots were removed and transferred to the reaction solution (50 μL; final concentration is 0.1 μM pigments, 1.5 μM transducin, 2.4 μM [γ-^{32}P]GTP precooled at 2 °C). Then the mixture was incubated at 25 °C for 2 min for the GTPase reaction, and the reaction was stopped with EDTA solution. The released P_i was collected by addition of activated charcoal and assayed by liquid scintillation counter (LS6000IC, Beckman). The activity of the pigment without irradiation was measured and subtracted as background. Aliquots of irradiated pigments (100 μL) were completely bleached with yellow light (>480 nm) in the presence of hydroxylamine (10 mM) to estimate the amounts of bleached pigments. The average and standard deviation were calculated for each point from at least three independent experiments. The activity immediately after the irradiation was measured from the sample mixed before the irradiation and was confirmed to be almost identical to that of pigment added to the reaction mixture after the irradiation. The linear relationship between the active pigment concentration in the sample and GTPase activity was confirmed using rhodopsin.

RESULTS

Batho Intermediate and Its Absorption Spectrum. Absorption maximum of chicken blue at 0 °C was 455 nm (data not shown), which was identical to that reported previously (Okano et al., 1989). When chicken blue was cooled to −196 °C, its spectrum was sharpened with a shift in its maximum to 458 nm (curve 1 in Figure 1A). Irradiation with blue light (436 nm) at this temperature caused a red-shift of absorption spectrum, indicating the formation of a batho-intermediate (cB-batho) (curve 2). Further irradiation caused a formation of a photo-steady-state mixture composed of original, its 9-cis product (cB-iso) and cB-batho (curves 2−8). The photoreversibility among chicken blue, cB-batho, and cB-iso was demonstrated in the inset of Figure 1B (curves 1′−4′).

Interestingly, absorption spectrum of the photo-steady-state mixture containing mainly cB-batho (curve 8 in Figure 1A) exhibits a maximum at 470 nm with prominent two shoulders at about 500 and 430 nm. Thus, to examine which product, cB-batho or cB-iso, contributes the structured absorption spectrum, we calculated the spectra of cB-batho and cB-iso using the procedure reported previously (Yoshizawa & Shichida, 1982; Imai et al., 1994). Since the amounts of chicken blue and cB-iso in the photo-steady-state mixture (curve 3′ in inset of Figure 1B) were 46 and 54%, respectively, the spectrum of cB-iso (curve 3 in Figure 1B) was calculated by subtracting the spectrum of 46% of chicken blue from that of the mixture. The spectrum of cB-batho

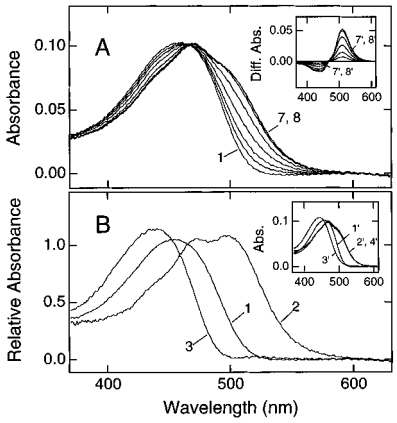

FIGURE 1: Photoreaction of chicken blue at −196 °C. (A) Formation of batho intermediate of chicken blue. The pigment/72% glycerol mixture was cooled to −196 °C (curve 1), followed by (the continuous) irradiation with blue light (436 nm) for 5, 10, 20, 40, 80, 160, and 320 s (curves 2−8) until the formation of a photo-steady-state mixture containing mainly cB-batho. (Inset) Difference spectra calculated by subtracting the spectrum before irradiation from those after irradiation (curves 2′−8′). (B) Calculated absorption spectra of photoproducts formed at − 196 °C. Absorption spectra of cB-batho (curve 2) and cB-iso (curve 3) were calculated from the spectra of the photo-steady-state mixtures that containing mainly cB-batho and cB-iso, respectively (curve 2′ and curve 3′ in inset, respectively). The photo-steady-state mixture containing mainly cB-iso was prepared by irradiating the photo-steady-state mixture containing mainly cB-batho (original state) (curve 2′) with yellow light (>480 nm) for 480 s. The absorbance at 455 nm of original chicken blue (curve 1) at 0 °C was adjusted to 1 OD.

(curve 2 in Figure 1B) was similarly calculated by subtracting the spectra of 27% chicken blue and 7% cB-iso from that of the photo-steady-state mixture containing mainly cB-batho (curve 8 in Figure 1A). cB-batho exhibited a structured spectrum having a λ_{max} at 499 nm with two shoulders at 473 and 433 nm, while cB-iso exhibited a broad spectrum with a maximum at 440 nm. Thus, it is clear that the spectrum of cB-batho contributes to the structured spectrum of the mixture. The structured spectrum of cB-batho was confirmed by three independent experiments using different samples.

Since Gaussian band-shape simulation with a spacing corresponding to the vibrational frequencies of polyene stretching modes (1500 cm^{-1}) reproduced the structured spectrum of cB-batho (data not shown), the cB-batho could be a single component. To examine it, cB-batho was photoconverted to chicken blue by irradiation of a longer wavelength tail of the spectrum of cB-batho with a >570 nm light and changes in absorbance at 500 and 450 nm were plotted as a function of irradiation time (Figure 2A). If cB-batho were a composite of numbers of species with different absorption maxima, some of them should have been selectively photoconverted by this irradiation. However, the time profiles at two wavelengths obtained by successive irradiation were indistinguishable from those observed in the conversion process from bathorhodopsin to rhodopsin (Figure 2B). Thus cB-batho is likely to be a single component.

Identification of BL, Lumi, Meta I and Meta II Intermediates. For estimation of the number of intermediates that exist in the bleaching sequence of chicken blue, the photo-steady-state mixture containing mainly cB-batho was warmed in a stepwise manner, and the spectra were measured (Figures 3 and 4). Four shifts in absorption spectrum were observed with the transition temperatures at −160, −110, −40, and −20 °C, indicating the presence of four intermediates besides

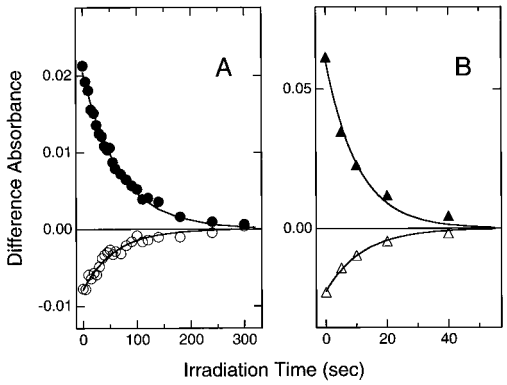

FIGURE 2: Kinetic profiles of the photoreaction of batho intermediates. The photo-steady-state mixture(s) containing mainly batho intermediate of chicken blue (A) or rhodopsin (B) was irradiated at −196 °C with orange light (>570 nm) or red light (>660 nm), respectively. The change of absorbance at 500 nm (closed circle) and 450 nm (open circle) in chicken blue, or (and) 550 nm (closed triangle) and 450 nm (open triangle) in rhodopsin were plotted against the irradiation time.

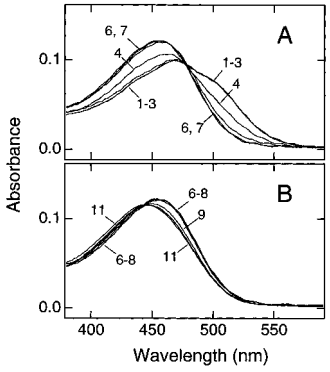

FIGURE 3: Thermal reaction of cB-batho and cB-BL. The photo-steady-state mixture containing mainly batho intermediate formed at −196 °C (curve 1) was warmed in a stepwise manner and the spectra were recorded. In panel A, absorption spectra were recorded at −180, −170, −160, −150, −140, and −130 °C (curves 2−7). In panel B, absorption spectra were recorded at −140, −130, −120, −110, −100, and −90 °C (curves 6−11). The spectral changes observed in panels A and B represent the batho-to-BL and BL-to-lumi transitions, respectively.

cB-batho. The final shift represented a formation of an intermediate having absorption maximum at about 380 nm, which was similar to those of meta II of rhodopsin, chicken red, and green. Thus, the fifth intermediate detected in this experiment should be assigned as meta II of chicken blue (cB-meta II). Since the transition temperatures for the fourth (Figure 4B) and third (Figure 4A) transitions were similar to those for meta I-to-meta II and lumi-to-meta I transitions in other visual pigments, we assigned the fourth and third intermediates as cB-meta I and cB-lumi, respectively. Thus, the second intermediate which was formed from cB-batho should be cB-BL. The conversion from cB-lumi to cB-meta I resulted in a red-shifted absorption spectrum, which was in marked contrast to rhodopsin, chicken red, and green. The unique shift might reflect the conformational changes of the protein moiety upon formation of meta I (see Discussion). It should be noted that we failed to observe interconversion of the intermediates by cooling the sample. Thus, the equilibrium among intermediates, if present, is little temperature dependent. It is in contrast to the case of meta I and meta II of rhodopsins (Matthews et al., 1963; Imai et al., 1994) and cRh-lumi and meta I (Imai et al., 1994).

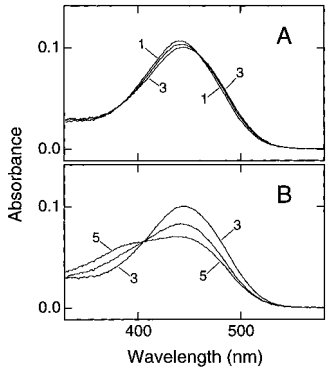

FIGURE 4: Thermal reaction of cB-lumi and cB-meta I. The chicken blue/56 % glycerol sample was irradiated and incubated at −40 °C (curve 1), followed by warming in a stepwise manner. In panel A, absorption spectra were recorded at − 40 (immediately after irradiation), −40 (16 h after irradiation), and −30 °C (curves 1−3). In panel B, absorption spectra were recorded at −30, −20, and −10 °C (curves 3−5). The spectral changes observed in panel A and B represent the lumi-to-meta I and meta I-to-meta II transitions, respectively.

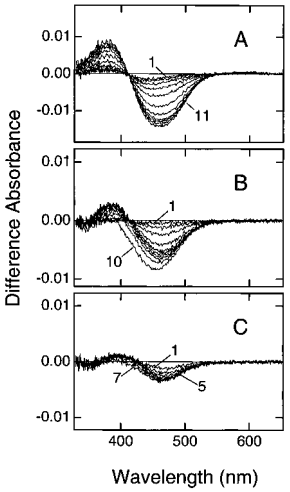

FIGURE 5: Thermal reactions of meta intermediates of chicken blue measured at −20 (A), −10 (B), and 0 °C (C) by time-resolved, low-temperature spectroscopy. Chicken blue/56% glycerol mixture was cooled to each temperature and irradiated with orange light (>520 nm) for 30 s. Absorption spectra were recorded immediately (0 min), 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, and 2048 min after the irradiation. Difference spectra calculated by subtracting the spectra recorded 2−2048 min after irradiation from that immediately after irradiation are shown (curves 1−11).

Thermal Decay of Meta Intermediates. Since our previous studies showed that meta II intermediates of chicken red and green decay more rapidly than metarhodopsin II, it was of interest to examine whether or not cB-meta II also decays rapidly. Thus, we investigated the decay process of cB-meta II by means of time-resolved low-temperature spectroscopy (Figure 5). When chicken blue was incubated at −20 °C after irradiation with orange light, an increase in absorbance at about 380 nm and a decrease of absorbance at about 450 nm were observed. These spectral changes are attributed to the formation process of cB-meta II from cB-meta I. Formation of another intermediate was observed as an increase of absorbance around 400 nm when the sample was incubated at −10 or 0 °C after the irradiation (panels B and C). Since the positive peak is located at a longer wavelength than that of meta II (panel A) and the increase of absorbance is countervailed by the decrease around 360 nm, the formed intermediate was assigned to meta III. The following

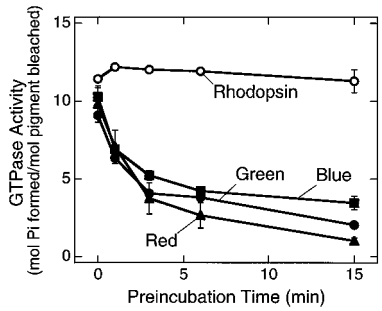

FIGURE 6: Thermal decay of active state of chicken rod and cone visual pigments monitored by time-resolved GTPase assay. The solution of chicken rhodopsin (open circle), red (triangle), green (closed circle), and blue (square) were irradiated at 2 °C with orange light for 30 s. After the indicated preincubation time in the dark at this temperature, 20 μL aliquots were removed and transferred to the reaction solution (50 μL; final concentration is 0.1 μM pigments, 1.5 μM transducin, 2.4 μM [γ-^{32}P]GTP). Then the GTPase activity was measured at 25 °C for 2 min. The activities were normalized to represent that they were induced by equivalent amounts of bleached pigments. Each points is the average of at least three independent experiments. Standard deviation in each point is shown as a vertical bar.

decrease of absorbance observed in Figure 5, panels B (curves 8−10) and C (curves 5−7), would be due to the decay of thermal equilibrium state between meta II and meta III to retinal and opsin. The kinetic profiles at 360 and 400 nm at −10 °C were fit by two single exponentials, and the time constants of cB-meta II and meta III were estimated to be 12 and 130 min, respectively. These are similar to those of cG-meta II and meta III (1.6 and 38 min) and much smaller than those of cRh-meta II and meta III (88 and 17 000 min, respectively).

Efficiency of Chicken Blue in Activating Transducin. Since it has been shown that meta II of rhodopsin (Emeis & Hofmann, 1981; Fukada & Yoshizawa, 1981; Bennett et al., 1982) and chicken red (Okada et al., 1994) activate transducin, the amount of transducin activated would be regulated by the thermal decay rate of meta intermediates (meta II and meta III). Thus, we have investigated the efficiency in activating transducin by examining GTPase activity of transducin as a function of preincubation time before addition of the irradiated visual pigments into the reaction mixtures containing transducin and GTP (Figure 6). When the pigments were irradiated in the presence of transducin and GTP, the differences in the activities induced by the pigments were within 10% of each other. Therefore, the catalytic turnover rates in transducin activation were similar in these pigments, which is consistent with the previous study using chicken red (Fukada et al., 1989b). On the other hand, the activities induced by the irradiated chicken blue and other cone pigments were greatly reduced as the preincubation time increased, while that by irradiated rhodopsin was not. These results strongly suggest a close relationship between decay rate of meta intermediates and efficiency in activating transducin.

DISCUSSION

In the present study, we have investigated the photobleaching process of chicken blue and its efficiency in activating G-protein. The results showed that chicken blue has a unique batho intermediate whose absorption spectrum exhibits vibrational fine structure. This is the first demonstration of vibrational fine structure in the spectrum of a batho intermediate formed from a pigment whose original spectrum does not exhibit vibrational fine structure. The present results also showed that chicken blue has meta II and meta III intermediates that decay with time constants similar to those of chicken red and green but much faster than those of rhodopsin. The time profile of the transducin activation of chicken blue is similar to those of chicken red and green but considerably different from that of rhodopsin. These results further indicate that one of the common properties of cone visual pigments is the faster decay of enzymatically active intermediates and exclude a possibility that the faster decay observed in chicken red and chicken green is an accidental event. Now, we will discuss the molecular events that occur during the bleaching processes of visual pigments and the process in activating G-protein on the basis of the acquired results.

Chicken Blue and Its Batho Intermediate. Chicken blue has an absorption maximum at 455 nm which is about 15 nm (750 cm^{-1}) red-shifted from that of a protonated Schiff's base in standard conditions (i.e., in methanol in the presence of Cl$^-$). According to site-directed mutagenesis experiments, several amino acid residues which are responsible for the red-shift of the spectra of the visual pigments have been identified (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990; Nakayama & Khorana, 1991; Merbs & Nathans, 1993; Chan et al., 1992; Asenjo et al., 1994). Among the residues, the tryptophan at position 265 (W265) and threonine at position 269 (T269) are conserved in chicken blue. Previous reports showed that these residues induce about 800 (W265) and 500 (T269) cm^{-1} red-shift of the maximum of rhodopsin, respectively (Nakayama & Khorana, 1991; Chan et al., 1992). The location of two residues one helical turn apart might lead to synergistic shifts in the absorption maximum. Thus, the difference in maximum between chicken blue and protonated Schiff's base could be explained by the presence of the residues in chicken blue. W265 and neighboring leucine 266 (L266) in rhodopsin were localized near the β-ionone ring of the retinylidene chromophore (Nakayama & Khorana, 1990; Zhang et al., 1994). Thus, these residues which induce the red-shift are likely to be situated near the β-ionone ring region of the chromophore in chicken blue. The rest of the chromophore in chicken blue may resemble the protonated Schiff's base in solution, except for its situation within a chiral cavity in the protein moiety as suggested in rhodopsin (Shichida, 1986). Relatively less interaction between polyene chain and protein moiety has been suggested in frog green rod pigment whose absorption maximum is about 440 nm (Loppnow et al., 1989).

Polyenes similar to retinal in the length of their double bond systems frequently show an absorption spectrum with vibrational fine structure, but 11-*cis*-retinal and animal visual pigments exhibit no vibrational fine structure. This is also the case with chicken blue. In visual pigments, the absence of vibrational fine structure is thought to arise from distortions at C_6-C_7 and $C_{12}-C_{13}$ single bonds due to steric hindrance between the C_5 methyl group and the C_8 proton and that between the C_{13} methyl group and the C_{10} proton (Smith et al., 1987). These distortions induce large Franck−Condon active vibronic intensities of low-frequency torsional modes that facilitate the loss of fine structure. Furthermore, electrostatic perturbation as well as steric interaction could

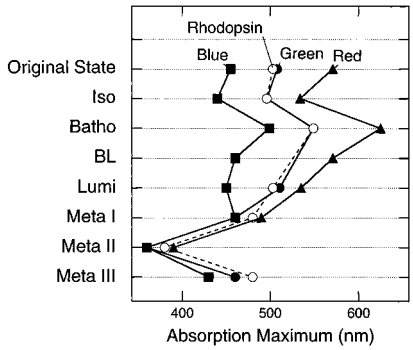

FIGURE 7: The shifts of absorption maxima of the intermediates appearing in the photobleaching process of chicken rod and cone visual pigments. Absorption maxima of the intermediates of chicken blue (square), red (triangle), green (closed circle), and rhodopsin (open circle) are plotted as a function of wavelength. The absorption maxima of intermediates of chicken red, green, and rhodopsin are reported previously (Kandori et al., 1990; Shichida et al., 1993; Imai et al., 1994; 1995). The temperatures at which the maxima were determined are −196 °C (iso, batho, BL, and lumi), −40 °C (meta I), −20 °C (meta II), and −10 °C (meta III) in chicken blue, green, and rhodopsin (Imai et al., 1994, 1995). In chicken red, the maxima of original and iso pigments at 0 °C and those of all the intermediates at 20 °C were plotted (Fukada et al., 1990; Kandori et al., 1990; Shichida et al., 1993). No BL was detected in chicken green and rhodopsin (Imai et al., 1995).

induce an inhomogeneous broadening of the spectra (Takahashi et al., 1990). Thus, the appearance of vibrational fine structure in the spectrum of cB-batho could be the loss of the steric hindrance and/or the conformational homogeneity caused by the isomerization of the chromophore. The absence of fine structure in the spectrum of cB-iso suggests that the intrinsic distortion originating from the 11-cis configuration is not the main factor that diminishes the fine structure in chicken blue system. Thus, the chromophore of cB-batho could have a more restricted C_6-C_7 conformation than those of cB-iso and original chicken blue. The sequence homology among visual pigments suggests that the chromophore binding site of chicken blue is similar to those of other visual pigments except for the presence or absence of those amino acid residues which regulate the absorption maxima. The absence of vibrational fine structure in the spectra of batho in rhodopsin, chicken green, and chicken red might be due to inhomogeneous broadening induced by a large electrostatic perturbation in these pigments relative to cB-batho, which has been suggested to be responsible for the greater red-shift of these pigments. In this context, it is of interest to examine whether or not the batho intermediates of other short wavelength sensitive visual pigments show vibrational fine structure.

Spectral Changes of Intermediates in the Bleaching Process of Chicken Blue. Using a method previously described (Yoshizawa & Shichida, 1982; Imai et al., 1995), we have estimated the absorption maxima of batho, BL, lumi, meta I, meta II, and meta III intermediates of chicken blue and its iso pigment, cB-iso. All the intermediates have their specific absorption maxima due to the different chromophore/opsin interactions in these intermediates. However, when the maximum values of the intermediates together with those obtained in chicken green, red, and rhodopsin are plotted, a relationship between the mechanism to regulate absorption maxima of intermediates and the conformational changes occurring in the course of bleaching is visualized (Figure 7). Namely, the maxima of batho, BL, and lumi intermediates in these pigments reflect the differences in maxima of original pigments, while those of meta I, meta II, and meta III converge to similar values. Since absorption maxima of pigments and their intermediates should be regulated by the amino acid residues situated near the chromophore in addition to the intrinsic properties of chromophore, these facts strongly suggest that the chromophore/opsin interaction greatly changes in the lumi-to-meta I transition. In other words, the amino acid residues which regulate the absorption maximum of original pigment do not change their positions significantly up to the formation of lumi intermediate, while they would part from the chromophore in meta I stage. This is consistent with the notion that large conformational changes occur in the transition from lumi to meta I (Shichida et al., 1991; Imai et al., 1994, 1995).

Thermal Decay Rate of Active State. In the present study, we investigated the decay kinetics of active state of chicken blue by spectroscopic and biochemical techniques and compared them with those of other cone and rod visual pigments. The result showed that the decay of active state of chicken blue is as fast as those of chicken red and green, and considerably faster than that of rhodopsin. Thus, it is confirmed that one of the common properties of cone visual pigments is the fast decay of active state.

Since the decay kinetics of the active state of chicken blue were observed by spectroscopic and biochemical techniques, a comparison between them provides detailed information about the intermolecular interaction between the visual pigment and transducin. The decay kinetics of active state of cone pigments monitored by the GTPase activity could be separated into two components. The fast component is largely complete after 3 min of incubation, while the slow component still remains after 15 min of incubation. The spectroscopic measurements revealed that the meta II-to-meta III transition occurs within several minutes, followed by the decay of the equilibrium state between meta II and meta III (Figure 5C). It should be noted that the decay kinetics of meta II and meta III in detergent solution present in this study are almost identical with those observed in PC liposome (data not shown). Therefore, the fast component observed by the biochemical method reflects the conversion process of meta II to the equilibrium state between meta II and meta III. The slow component reflects the decay process of the equilibrium state between meta II and meta III to *all-trans*-retinal and opsin. Thus, we concluded that the intermediate which activates transducin should be meta II. Because of limited time resolution of the biochemical measurements, we are unable to exclude the possibility that meta III of cone pigments activates transducin.

Recently, we showed that the decay rate of meta II of rhodopsin was accelerated by the single replacement of glutamate at position 122 (E122) by the residue present in chicken red (isoleucine) or green (glutamine) (Imai et al., 1997). In addition, present results clearly showed that meta II of chicken blue, which has a methionine at the corresponding position, decays with a time constant similar to those of chicken red and green. These facts indicate the importance of the residue at position 122 on the thermal stability of meta II. Our preliminary studies on chicken violet, which has leucine instead of glutamate at this position, also indicated that it exhibits faster decay of active state (H. Imai, et al., unpublished result). This is consistent with the recent report on *Xenopus* violet-sensitive pigment expressed

in COS1 cells (Starace & Knox, 1997). Although the mechanism of stabilization of meta II by E122 is not fully understood, these results strongly suggest that the faster decay of the enzymatically active state is common in cone visual pigments, independent of their spectral sensitivity.

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