Purification of Cone Visual Pigments from Chicken Retina†

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ABSTRACT: A novel method for purification of chicken cone visual pigments was established by use of a 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate–phosphatidylcholine (CHAPS–PC) mixture. Outer segment membranes isolated from chicken retinas were extracted with 0.75% CHAPS supplemented with 1.0 mg/mL phosphatidylcholine (CHAPS–PC system). After the extract was diluted to 0.6% CHAPS, it was loaded on a concanavalin A–Sepharose column. Elution from the column with different concentrations of methyl α-mannoside yielded three fractions: the first was composed of chicken violet, blue, and red in roughly equal amounts, the second predominantly contained chicken red, and the third was rhodopsin with a small amount of chicken green, which was separated from rhodopsin by DEAE-Sepharose column chromatography. Since CHAPS has little absorbance at both ultraviolet and visible regions, we could demonstrate the absolute absorption spectra of chicken red (92%) and rhodopsin (>96%) in these regions. The maximum of the difference spectrum between either chicken red or rhodopsin and its photoproduct (*all-trans*-retinal oxime plus opsin) was determined to be 571 or 503 nm, respectively. Although chicken green was contaminated with a small amount of rhodopsin having a similar spectral shape, the maximum of its difference spectrum was located at 508 nm by taking advantage of the difference in susceptibility against hydroxylamine between these pigments. Although chicken blue and chicken violet were minor pigments present in the first fraction from the concanavalin A column, their maxima in the difference spectra were determined to be at 455 and 425 nm, respectively, by a partial bleaching method. When the difference spectra were plotted on a scale of the fourth root of the wavelength and the maxima were shifted on the scale so as to coincide with each other, the spectra of the pigments except for chicken violet were superimposable with each other in the longer wavelength region. On the assumption that the difference spectrum of chicken violet was distorted by the absorption of retinal oxime, the difference spectrum was corrected to a maximum of 415 nm. Thus, we could convincingly identify the four kinds of cone pigments in vitro and demonstrate their absorption maxima. These values were in good agreement with those determined microspectrophotometrically rather than those reported in digitonin, suggesting that the conformations of pigments in the CHAPS–PC system may be closer to the native ones than those in the digitonin system.

Rod photoreceptor cells, responsible for scotopic vision under twilight, contain only one visual pigment, rhodopsin, with a high photosensitivity, while cone photoreceptor cells take part in photopic vision to discriminate intensities of light over a wide range in a short time span. In many vertebrates, cone cells can be functionally classified into several types according to differences in absorption spectra of photoreceptive pigments, providing a basis for color discrimination. To clarify the differences in mechanism between photopic and scotopic vision, comparative studies on both systems have been carried out by using morphological, psychological, physiological, spectrophotometrical, and biochemical techniques. Recent electrophysiological experiments on cones have revealed the existence of a cGMP-sensitive cationic channel in the plasma membrane (Haynes & Yau, 1985; Cobbs et al., 1985). In addition, it has been demonstrated biochemically that cones contain two proteins essential for the cGMP cascade: transducin (Lerea et al., 1986; Fukada & Akino, 1986) and cGMP phosphodiesterase (Gillespie & Beavo, 1988). These studies suggest that the cone transduction mechanism is similar to the rod one [for a review, Hurley (1987)]. However, none of these biochemical studies have demonstrated the molecular basis of the physiological difference between rod and cone cells. Since it is assumed that the difference in photosensitivity between rod and cone cells may be, at least partly, based on the difference between photosensitivities of the photoreceptive pigments themselves, it is essential to isolate each of cone visual pigments in a physiologically active form as a first step for examining these problems.

Some years ago four kinds of cone visual pigments were solubilized with digitonin from chicken retinas, from which chicken red (iodospin)[1] was isolated by column chromatographic procedures (Fager & Fager, 1982; Yen & Fager, 1984). Since the first report on an extraction of chicken red (Wald, 1937), only digitonin has been used as the detergent to solubilize cone pigments, because the use of other detergents (1% cetyltrimethylammonium bromide, 1% Emulphogene BC-720, 1% Triton X-100, 1% sodium cholate, deoxycholate,

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[1] The term "iodopsin" is a specific name of chicken red sensitive cone pigment (Wald et al., 1955). Since there are no specific terms of other cone pigments, it would be better to use a name derived from both the animal species and the color characterizing the spectral sensitivity of the pigment as Yen and Fager (1984) proposed. In this paper, we will call the chicken red sensitive cone pigment chicken red instead of iodopsin.

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Ammonyx-LO) caused denaturation of the cone pigments (Fager, 1975; Fager & Fager, 1982). Although digitonin is surely a good detergent for solubilization of visual pigments in stable forms, it has several disadvantages; one of the most significant ones is that it hinders biochemical investigations of cone pigments, because it is difficult to remove by dialysis or other means. In order to elucidate the transduction process in cones, it is necessary to prepare functionally active pigments without forming any large micelles of a detergent. Thus, we focused our study on extracting cone pigments using a detergent other than digitonin.

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)[2] has a higher cmc value (0.49%) and a lower micelle molecular mass (6150 Da; Hjelmeland & Chrambach, 1984) than digitonin [0.09% (Matsumoto et al., 1978) and 70000 Da (Hjelmeland & Chrambach, 1984)] and is easily removed by dialysis.

In this paper, we report a novel method to extract rod and cone visual pigments in stable forms from chicken retinas by CHAPS in the presence of phosphatidylcholine. Subsequent chromatographic procedures led us to demonstrate convincingly the existence of four kinds of cone pigments and their spectral properties.

Materials and Methods

Chemicals. Phenylmethanesulfonyl fluoride ($PhMeSO_2F$), aprotinin, leupeptin, *all-trans*-retinal, and L-α-phosphatidylcholine (PC) from fresh egg yolk (type XI-E) were products of Sigma. ConA–Sepharose and DEAE-Sepharose CL-6B 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 buffers was adjusted to 6.6 at 4 °C with NaOH. Buffer P (for preparation) contained 50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM $PhMeSO_2F$, 50 kallikrein inhibitor units/mL aprotinin, and 4 μg/mL leupeptin. Buffer E (for extraction) was buffer P supplemented with 0.75% CHAPS, 1.0 mg/mL PC, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. Buffer A (for affinity column) was buffer P supplemented with 0.6% CHAPS, 0.8 mg/mL PC, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. Buffer D-20 (for DEAE column) contained 20% (w/v) glycerol, 0.6% CHAPS, 0.8 mg/mL PC, 50 mM HEPES, 1 mM DTT, 0.1 mM $PhMeSO_2F$, 50 kallikrein inhibitor units/mL aprotinin, and 4 μg/mL leupeptin.

Isolation of Chicken Retinas. Chicken heads were brought to the laboratory in a light-tight ice box within a few hours after death. All procedures were performed at 4 °C unless otherwise stated. Dissection of the eyeballs and further manipulations before adding 11-*cis*-retinal (see below) were carried out under red light (>640 nm), which only partially bleached chicken red. After cutting off the pecten, the eyecup was kept in buffer P until the retina detached spontaneously. The retinas were collected in a tube, pelleted by centrifugation at 16000*g* for 10 min, and stored at −80 °C until use.

Isolation of Outer Segments. Frozen retinas were thawed and vigorously shaken for 1 min in buffer P containing 40% (w/v) sucrose (approximately 0.5 mL per retina) in order to detach rod and cone outer segments from retinas. After centrifugation at 20000*g* for 20 min, the supernatant with some floating material was collected. Then the pellet was mixed with the sucrose solution, followed by centrifugation, and the supernatant was collected. This step was repeated once more. All supernatants thus collected were diluted with an equal volume of buffer P and centrifuged at 20000*g* for 30 min to sediment the outer segments. To remove oil droplets and sucrose, the outer segments were homogenized in buffer P with a Teflon homogenizer (10 strokes) and centrifuged (20000*g*, 30 min). The supernatant was discarded. This washing was repeated three to six times until the supernatant became colorless.

Extraction of Outer Segment Proteins. The outer segment pellet thus obtained was suspended with buffer E (approximately 0.33 mL per retina) containing a 2-fold molar excess of 11-*cis*-retinal over the amount of R-photopsin[3] and homogenized with a Teflon homogenizer (30 strokes) for extraction of the visual pigments. Then the homogenate was kept in the dark for 30 min to regenerate chicken red. As mentioned before, pigments other than chicken red had not been bleached, because all the manipulations had been performed under red light (>640 nm). Subsequent operations were carried out in complete darkness or under dim red light (>680 nm). After centrifugation (110000*g*, 40 min), the supernatant was collected. Buffer P (1 volume) supplemented with 1 mM $MnCl_2$ and $CaCl_2$ was added to the supernatant (4 volumes) to decrease the concentration of CHAPS to 0.6%. The diluted preparation, tentatively named "CHAPS–PC extract", was applied to a ConA–Sepharose column (see below). In a typical experiment in which visual pigments were extracted from 546 retinas, 270 mL of supernatant was obtained and diluted to yield 338 mL of CHAPS–PC extract.

Column Chromatographies. The bed volume of the ConA–Sepharose affinity column was 50–100 mL, depending on the amount of starting material; 0.25–1.0 mL of ConA–Sepharose was used for the CHAPS–PC extract from 10 retinas. Usually 0.02–0.03 ΔOD·mL chicken red,[4] 0.03–0.04 ΔOD·mL rhodopsin, and less than 0.01 ΔOD·mL other cone pigments were obtained from one retina. Approximately 0.7–2.0 ΔOD·mL visual pigment can be adsorbed to 1 mL ConA–Sepharose. When visual pigments were extracted from 546 retinas, a ConA–Sepharose column (16 mm × 270 mm) washed in advance with 10 bed volumes of 1 M NaCl was equilibrated with 5 bed volumes of buffer A at a flow rate of 14 mL/h. The CHAPS–PC extract (338 mL) was loaded on the column and washed with 8 bed volumes of buffer A to remove unbound material including oil droplet components. Then the bound proteins were eluted in order with 2.5 bed volumes of 1.5 mM methyl α-D-mannoside (methyl α-mannoside), 1.6 bed volumes of 5 mM methyl α-mannoside, and 2 bed volumes of 100 mM methyl α-mannoside in buffer A. Throughout these procedures (sample loading, washing, and elution), the flow rate was fixed at 8 mL/h. The absorbance of eluate was continuously monitored at 280 nm. The fractions eluted with methyl α-mannoside from the ConA column were mixed with an equal volume of buffer D-20 containing 40%

[2] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $PhMeSO_2F$, phenylmethanesulfonyl fluoride; ConA, concanavalin A; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PC, L-α-phosphatidylcholine from fresh egg yolk; cmc, critical micellar concentration; OG, *n*-octyl β-D-glucoside; methyl α-mannoside, methyl α-D-mannoside; Da, dalton(s); λ_{max}, absorption maximum in visible region.

[3] The opsin moiety of chicken red (iodopsin) which is responsible for photopic vision was called photopsin. Here, we propose to use "photopsin" prefixed an initial letter of color characterizing the spectral sensitivity of a cone pigment for expression of the opsin moiety. For example, R-photopsin means the opsin moiety of chicken red.

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

(w/v) glycerol instead of 20% (w/v) to give a final glycerol concentration of 20% (w/v).

After the supplement of glycerol, all the eluates with 100 mM methyl α-mannoside from the ConA column (fractions 11–13 in Figure 3) were mixed together. Then they were concentrated to 10 mL by means of Amicon ultrafiltration cell (Model 8050) with a YM-30 membrane filter and subsequently desalted by limited dialysis in the ultrafiltration cell against buffer D-20. Remarkably, the addition of glycerol prevented the pigments from denaturation during these processes (see Results). The desalted material (22 mL) was applied on a DEAE-Sepharose column (16 mm × 300 nm) which had been equilibrated with 6 bed volumes of buffer D-20. After washing the column with 2 bed volumes of buffer D-20, the bound proteins were eluted successively with 100 and 400 mM NaCl in buffer D-20 at a flow rate of 14 mL/h. The eluates from the columns were monitored continuously by measuring absorbance at 280 nm.

Spectrophotometric Measurements. (*i*) *Samples.* In a preliminary experiment, we examined the stabilities of CHAPS-solubilized cone pigments against hydroxylamine and confirmed that all the cone pigments except for chicken green were stable in the presence of 10 mM hydroxylamine (hydroxylamine hydrochloride was adjusted to pH 6.6 with NaOH) at 4 °C, where only chicken green was bleached very slowly: The amount of bleached chicken green was less than 6% within the period of the spectrophotometric measurement (3–4 h). Therefore, we always added 10 mM hydroxylamine to each sample at 4 °C.

(*ii*) *Spectrophotometric Equipment.* All the absorption spectra were recorded by a Hitachi Model 330 spectrophotometer, from which the data (absorbance at each nanometer) were transferred to a personal computer 9801VM (NEC Co. Ltd.) and analyzed. The sample in an optical cell (volume, 0.4 mL; width, 2 mm; light path, 1 cm) was kept at a constant temperature (usually at 4 °C). The moisture on the cell surface was removed by blowing nitrogen gas. The sample was irradiated in the sample compartment of the spectrophotometer. The light source for irradiating the sample was a 1-kW tungsten–halogen lamp (Sanko Co. Ltd.). To remove heat from the irradiation light, a glass optical cell filled with distilled water (light path 5 cm) was placed between the light source and a filter (see below).

(*iii*) *Partial Bleachings.* The amount of each visual pigment in the sample was estimated by the partial bleaching method. A sample thought to contain more than one kind of visual pigment was irradiated with long-wavelength light by which only the visual pigment lying at the longest wavelengths could be bleached. After completion of bleaching of this pigment, the wavelength of irradiating light was shifted to shorter wavelengths. Then the sample was reirradiated, until the second pigment was completely bleached. Such a bleaching was repeated step by step until all the pigments in the sample had been belached. In each irradiation, the following combinations of a cutoff filter (Toshiba Co. Ltd.) and irradiation times for complete bleaching were used: VR68 (>660 nm, deep red light) for 40 min for bleaching of chicken red; VR61 (>590 nm, red light) for 20 min for both chicken green and rhodopsin; VO54 (>520 nm, orange light) for 10 min for chicken blue; VY50 (>480 nm, yellow light) for 5 min for chicken violet. After bleaching of chicken red by irradiation with the deep red light for 40 min, another irradiation (>660 nm, 40 min) was repeated to confirm the absence of chicken red in the sample. Such an irradiation for confirmation was carried out for each pigment.

Results

Conditions for Extraction of Pigments. In solubilizing a visual pigment, there are several criteria for finding a suitable detergent and conditions: the first is that the pigment solubilized should be stable in the dark, and the second is that an opsin solubilized can bind 11-*cis*-retinal to regenerate the pigment. Since a condition that does not satisfy the first criterion does not satisfy the second one, we began to look for a detergent that does not inhibit the regeneration of photopsin to a pigment in the presence of 11-*cis*-retinal.

Chicken outer segments were solubilized in the light with various concentrations of *n*-octyl β-D-glucoside (OG) or CHAPS dissolved in buffer P. When OG was used at the concentration above 0.8% (just above the cmc of OG), no chicken red was regenerated from the extract by the addition of 11-*cis*-retinal. For lowering the concentration of OG, the extract with 0.8% OG was diluted with buffer P. After addition of 11-*cis*-retinal, the extract became so turbid that spectrophotometric measurements could not be precisely performed.

On the other hand, extracts with CHAPS at concentrations above 1.0% displayed no regeneration of chicken red. The extract with 0.75% CHAPS regenerated a small amount of chicken red, but it gradually decomposed in the dark. Moreover, when the extract containing the regenerated chicken red was applied to the ConA column in the dark (even in the presence of glycerol; see below), no chicken red was eluted from the column by the addition of methyl α-mannoside. This observation indicated that chicken red was completely denatured in the course of ConA–Sepharose column chromatography by removal of endogenous lipid. In order to stabilize chicken red in the presence of CHAPS, PC was dissolved to give a final concentration of 1.0 mg/mL in the buffer for extraction (0.75% CHAPS in buffer P). This mixture brought a significant stabilization of chicken red. Addition of PC at a concentration higher than 1.0 mg/mL to the extraction buffer (0.75% CHAPS in buffer P) disturbed an effective solubilization.

Concentration of CHAPS after Extraction for Subsequent Column Chromatographies. In order to find the optimal concentration of CHAPS in the presence of PC during the long purification procedures, the effect of CHAPS concentration on the stability of R-photopsin was studied. R-Photopsin was extracted with a mixture of 0.75% CHAPS and 1.0 mg/mL PC dissolved in buffer P, and the extract was diluted with buffer P to lower the concentrations of CHAPS to 0.6%, 0.55%, or 0.5%. Then the 0.75% CHAPS extract and the diluted ones were incubated for 12 h at 20 °C. Before and after the incubation, the regenerability of R-photopsin in each sample was compared by addition of 11-*cis*-retinal. We found only a small difference in regenerability of R-photopsin among these samples at CHAPS concentrations below 0.6% [0.6%, 0.55%, and 0.5%; percentage of regenerability after incubation for 12 h to that before the incubation (residual regenerability) was approximately 50%], while a larger amount of R-photopsin lost the regenerability without dilution (0.75%; residual regenerability was less than 20%). Since this stable range (0.5%–0.6% of CHAPS) is close to the cmc value of CHAPS (0.49%; Hjelmeland & Chrambach, 1984), the higher concentration of CHAPS (0.6%) was employed to prevent aggregation of pigments. Thus, all the buffers used for further purification procedures contain 0.6% CHAPS and 0.8 mg/mL PC.

Stabilities of Visual Pigments and Their Opsins. The thermal stabilities of chicken red, rhodopsin, and their opsin

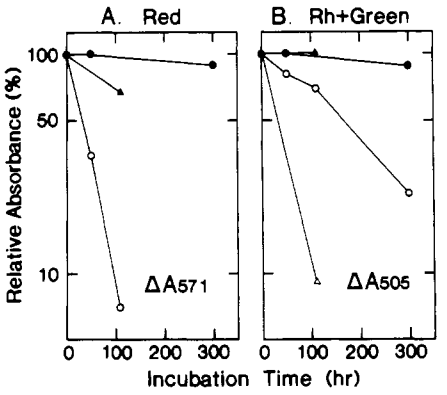

FIGURE 1: Comparison of the thermal stabilities of a pigment and its opsin. The CHAPS–PC extract ([CHAPS] = 0.6%, [PC] = 0.8 mg/mL; obtained from 15 retinas) supplemented with 20% (w/v) glycerol (for the effect of glycerol, see Figure 2) was irradiated with yellow light (>480 nm) for 10 min for complete bleaching of the pigments into *all-trans*-retinal and opsins. The extract was loaded on a ConA–Sepharose column (5 mm × 50 mm) in the light, and then the opsins were completely eluted with 200 mM methyl α-mannoside in buffer A containing 20% (w/v) glycerol. Immediately after the eluate was divided into eight aliquots (1.0 mL each), four of them were mixed with molar excess of 11-*cis*-retinal over the opsins and kept in the dark for 30 min at 4 °C (three closed circles) or 20 °C (one closed triangle). Before (0 h) or after the incubation, the absorbances of chicken red (panel A) and a mixture of rhodopsin and chicken green (panel B) in the aliquots were estimated by the partial bleaching method. Relative absorbance near the maximum (panel A, 571 nm for chicken red; panel B, 505 nm for a mixture of chicken green and rhodopsin) was plotted on a logarithmic scale (ordinate) against the incubation time (abscissa). The other four aliquots were kept in the light without addition of 11-*cis*-retinal at 4 °C (three open circles) and 20 °C (one open triangle). After incubation for indicated time, the same amount of 11-*cis*-retinal as described above was added and kept in the dark at 4 °C for 30 min. The absorbances of regenerated pigments were estimated similarly by the partial bleaching method. No chicken red was regenerated in the aliquots that had been incubated in the light either at 20 °C for 100 h or at 4 °C for 300 h (not plotted in panel A).

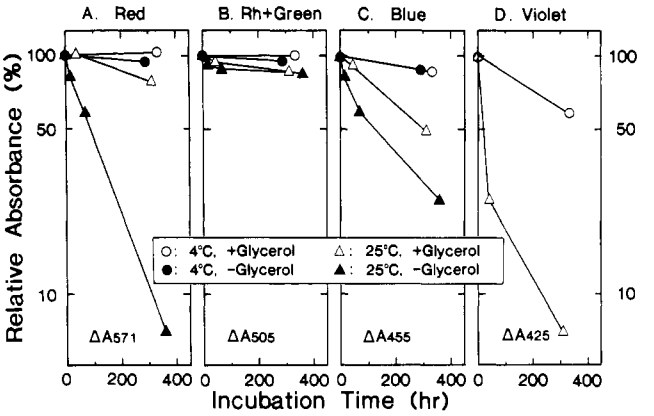

FIGURE 2: Effect of glycerol on the thermal stabilities of chicken visual pigments. The CHAPS–PC extract ([CHAPS] = 0.6%, [PC] = 0.8 mg/mL; see Materials and Methods) obtained from 28 retinas was loaded on a ConA–Sepharose column (10 mm × 50 mm) in the dark, from which cone pigment enriched fractions were eluted with 5 mM methyl α-mannoside at a flow rate of 3.3 mL/h. The eluate was divided into two portions, one of which was mixed with the equal volume of buffer A containing 40% (w/v) glycerol [final concentration 20% (w/v) glycerol]. Another portion was mied with an equal volume of buffer A without glycerol. They were further divided into two portions, respectively. Then, the four portions were incubated either at 25 or 4 °C. At appropriate times, a small aliquot (0.4 mL) was transferred from each portion to an optical cell to measure the absorbance of each pigment remaining in the sample by the partial bleaching method. Relative absorbance near the maximum (panel A, 571 nm for chicken red; panel B, 505 nm for a mixture of chicken rhodopsin and green; panel C, 455 nm for chicken blue; panel D, 425 nm for chicken violet) was plotted on a logarithmic scale (ordinate) against the incubation time (abscissa). Since it is impossible to separate chicken green (λ_{max} 508 nm) from rhodopsin (λ_{max} 503 nm) by the partial bleaching method, the sum of both pigments (absorbance at 505 nm) was shown in panel B.

moieties after being eluted from the ConA–Sepharose column were compared with one another (see legend to Figure 1). Figure 1A shows that chicken red (closed circles) is more stable than R-photopsin (open circles) at 4 °C; that is, only a small amount (less than 10%) of chicken red was thermally bleached during the incubation for 300 h at 4 °C, while more than 90% of R-photopsin lost its regenerability within 108 h at 4 °C. Similar results were obtained for a mixture of rhodopsin and chicken green (Figure 1B). Since the ratio of rhodopsin to chicken green in the sample used in this experiment was about 10:1, the points plotted in panel B represent predominantly rhodopsin (closed symbols) or scotopsin (open symbols). Thus, as is well-known, the pigments are obviously far more stable than their opsin moieties. Hereafter, purification procedures were performed under the safety light after the pigments were regenerated by addition of 11-*cis*-retinal.

Stabilization by Glycerol. Next, the stabilizing effect of glycerol on each pigment was examined by use of eluates from a ConA–Sepharose column (see legend to Figure 2). As shown in Figure 2, 20% (w/v) glycerol effectively stabilized both rod and cone pigments. This effect was particularly obvious on chicken red (panel A). In panel B, only a small effect of glycerol on a mixture of rhodopsin and chicken green was observed. This result does not always indicate that chicken green is more stable than the other cone pigments because this fraction contains predominantly rhodopsin. A little degradation without glycerol (panel B, triangles) may reflect the denaturation of chicken green. In fact, the other three kinds of cone pigments were unstable in the absence of glycerol, which was stabilized by addition of 20% (w/v) glycerol (panels A, C, and D). However, chicken violet bleached gradually in the dark even in the presence of glycerol (panel D). Although glycerol has a remarkable ability to stabilize visual pigments as shown in Figure 2, deletion of glycerol in buffer A during the ConA column chromatography resulted in better separation of each visual pigment. Thus, we concluded that glycerol should be added to the sample after the ConA column chromatography.

ConA–Sepharose Column Chromatography. (*i*) *CHAPS–PC Extract.* In the course of extraction of visual pigments from the chicken outer segments obtained from 546 retinas, 270 mL of a crude preparation extracted with CHAPS–PC was obtained, to which 68 mL of buffer P was added, which decreased the CHAPS and PC concentrations to 0.6% and 0.8 mg/mL, respectively (CHAPS–PC extract). The amounts of chicken red, chicken blue, and a mixture of rhodopsin and chicken green were 15.2, 4.4, and 19.5 ΔOD·mL, respectively, and their yields were 0.028, 0.008, and 0.036 ΔOD·mL/retina. The amount of chicken violet in the CHAPS–PC extract could not be estimated exactly because the content of chicken violet was very low in the extract, and also it contained an excess amount of 11-*cis*-retinal having a similar absorption spectrum. Since the first extract contained more than 90% of the cone pigments and 70% of the rhodopsin in the preparation, further extraction was not performed.

(*ii*) *Short-Wavelength Pigments.* The CHAPS–PC extract was loaded on the ConA–Sepharose affinity column, from which six fractions (22 mL each) were eluted with 1.5 mM methyl α-mannoside dissolved in buffer A, as shown in Figure 3. Fraction 1 contained a large amount of UV-absorbing materials without any visual pigment. Fraction 2 contained short-wavelength pigments, in which the relative content of

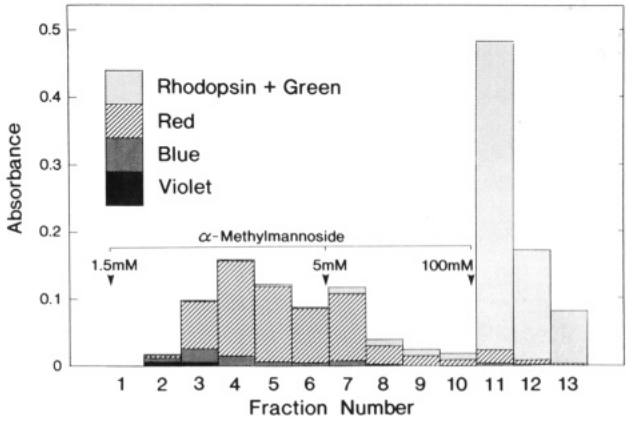

FIGURE 3: Elution profile of the CHAPS–PC extract from ConA–Sepharose affinity column chromatography. The chromatographic procedures are described in the text. Partial bleaching of each fraction (22 mL) was used to estimate the amount of each visual pigment. The amount of each pigment except for chicken violet was expressed as absorbance at the maximum of the difference absorption spectrum (505 nm for mixtures of rhodopsin and chicken green, 571 nm for chicken red, 455 nm for chicken blue). The amount of chicken violet was calculated from the difference absorbance at 425 nm multiplied by the $1/P2$ factor (=1.19; see legend to Figure 9).

chicken violet (38%) was highest among all the fractions. The difference absorption maximum of chicken violet was determined to be 425 nm by the partial bleaching of this fraction (Figure 4A). Fraction 3 displayed a peak in the elution of chicken blue. The difference absorption maximum of chicken blue was determined to be 455 nm by the partial bleaching of this fraction (Figure 4B).

(*iii*) *Chicken Red.* In fractions 4–6 the relative content of chicken red significantly increased, indicating that chicken red bound to the column with higher affinity than the short-wavelength pigments. Thus, chicken red was recovered with high purity in the later fractions. For instance, fraction 5 was composed of more than 92% of chicken red, 2% of a mixture of rhodopsin and chicken green, and 6% of chicken blue (Figure 4C). The content of chicken red in the corresponding fractions in several experiments ranged from 90% to 96%. The total recovery of chicken red through the ConA column was 86%. The spectrum of chicken red in fraction 5 showed the ratio between the absorbances at 280 nm and at 571 nm to be about 2.9 (Figure 4C, curve 1). This is the first demonstration of the absolute absorption spectrum of mainly chicken red (92%) not only in the visible but also in the UV region.

An SDS–PAGE analysis of fraction 5 displayed three major bands of 36, 70, and 110 kDa, which may correspond to monomer, dimer, and trimer of chicken red, respectively (Figure 5, lane R).

(*iv*) *Chicken Green and Rhodopsin.* For further elution from the ConA–Sepharose column (Figure 3), the concentration of methyl α-mannoside was increased to 5 mM in hope of complete elution of any chicken red remaining on the column. Though we failed to get a pigment fraction without any chicken red, the later fractions (9 and 10) contained no chicken blue. Finally, as the concentration of methyl α-mannoside was increased to 100 mM, a mixture of chicken green and rhodopsin was eluted as main components in fractions 11–13 (Figure 3). The three fractions were mixed together for further purification (see below). The mixture was composed of 1.54 ΔOD·mL chicken green and 14.7 ΔOD·mL rhodopsin with a little contamination (0.64 ΔOD·mL) of chicken red. (The method for estimating the amounts of these pigments is described in the next section.) The total recovery

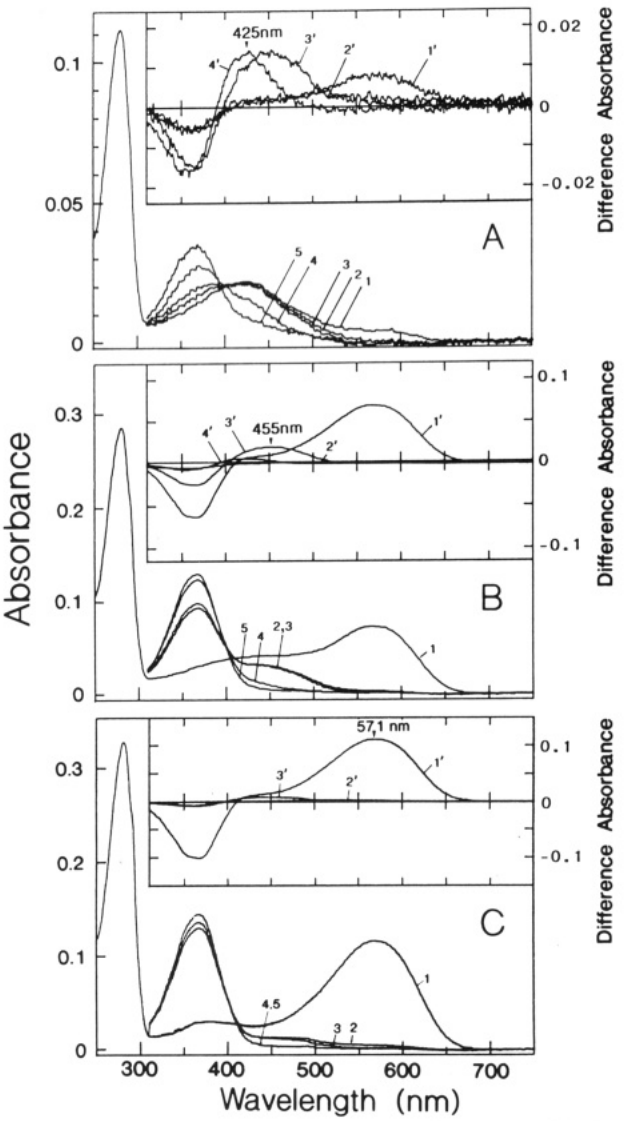

FIGURE 4: Determinations of difference absorption maxima of chicken violet, blue, and red by the partial bleaching method. Spectral changes by the partial bleaching of typical fractions (fractions 2, 3, and 5 in Figure 3) eluted from the ConA–Sepharose column are shown in panels A, B, and C, respectively. Each fraction was sequentially bleached by light at different wavelengths (see Materials and Methods). Curve 1: Absorption spectrum of the fraction before irradiation. Curves 2, 3, 4, and 5 are the spectra after sequential irradiations with deep red light (>660 nm, for 80 min), red light (>590 nm, for 40 min), orange light (>520 nm, for 20 min) and yellow light (>480 nm, for 10 min), respectively. Insets: Difference spectra calculated from those before and after the sequential irradiations. Curve 1′ = curve 1 − curve 2 (chicken red), curve 2′ = curve 2 − curve 3 (a mixture of rhodopsin and chicken green), curve 3′ = curve 3 − curve 4 (chicken blue), and curve 4′ = curve 4 − curve 5 (chicken violet). Fraction 2 was composed of 19% chicken red, 10% of a mixture of rhodopsin and chicken green, 33% chicken blue, and 38% chicken violet. Fraction 3 was composed of 71% chicken red, 2% of a mixture of rhodopsin and chicken green, 20% chicken blue, and 7% chicken violet. Fraction 5 was composed of 92% chicken red, 2% of a mixture of rhodopsin and chicken green, and 6% chicken blue. Since fraction 5 contained no chicken violet, curve 4′ was not shown in panel C.

of a mixture of rhodopsin and chicken green was 87% through the ConA column.

DEAE-Sepharose Column Chromatography. (*i*) *Chicken Green.* To a mixture of fractions 11–13 from the ConA column was added 20% glycerol, and they were concentrated, desalted, and then applied to the DEAE-Sepharose column as described under Materials and Methods. Chicken green passed through the column without adsorption and recovered

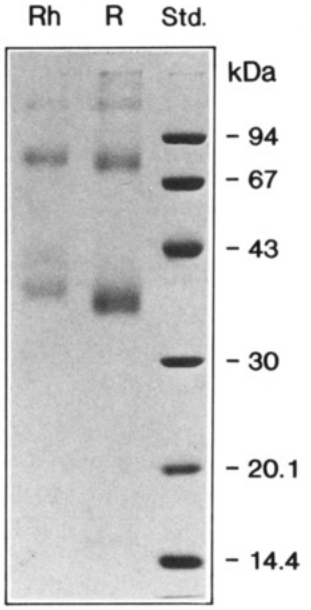

FIGURE 5: SDS–PAGE analyses of purified chicken red and rhodopsin. The SDS–PAGE was performed according to Laemmli's method (Laemmli, 1970) using 13% polyacrylamide. One of the fractions eluted with 1.5 mM methyl α-mannoside from the ConA column (fraction 5 in Figure 3) and the eluate with 100 mM NaCl from the DEAE column (the shaded part of the second peak fraction in Figure 6) were used as samples of chicken red (lane R) and rhodopsin (lane Rh), respectively. The samples containing 8.8×10^{-3} ΔOD·mL of the pigments were incubated with 2% SDS, 2% β-mercaptoethanol, and 2 mM EDTA at room temperature for 12 h and subjected to the analysis. Molecular masses of chicken red (lane R) and rhodopsin (lane Rh) were estimated to be 36 and 37 kDa, respectively. Additional bands at molecular masses of about 70 and 110 kDa detected in both lanes could correspond to dimers and trimers, respectively, because these bands were increased when the samples were boiled in the presence of SDS presumably due to the enhancement of the aggregation. Molecular mass standards (lane Std) were products of Pharmacia LKB Biotechnology Inc.

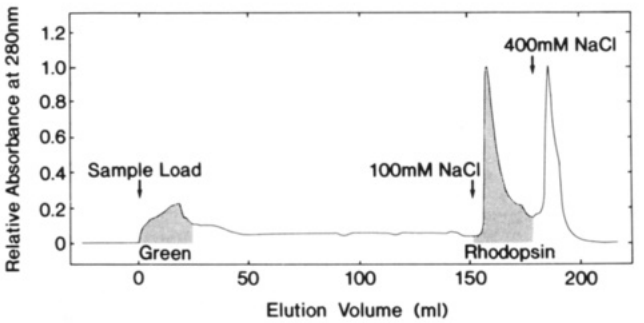

FIGURE 6: Elution profile of chicken green, rhodopsin, and other proteins by DEAE-Sepharose anion-exchange column chromatography. A mixture of chicken green and rhodopsin (the eluate from the ConA column; fractions 11–13 in Figure 3) was supplemented with glycerol [final concentration 20% (w/v)], concentrated, desalted, and then applied to the DEAE-Sepharose column as described in the text. The column was washed with 120 mL of buffer D-20, followed by successive elutions with 100 and then 400 mM NaCl dissolved in buffer D-20. The eluate was monitored continuously by absorbance at 280 nm.

in the first peak (Figure 6). Some portion of chicken green eluted with retardation as revealed by a long tail after the first fraction (25–155 mL). In order to evaluate the precise amount of chicken green in these fractions, they were mixed with hydroxylamine in a final concentration of 10 mM. The use of the hydrochloride form of hydroxylamine (the pH was adjusted with NaOH) will convert the chloride-free form of chicken red (Knowles, 1976), if any, into the chloride-bound form. Then the sample was kept in the dark at 23 °C. Since chicken rhodopsin is stable against hydroxylamine (Wald et

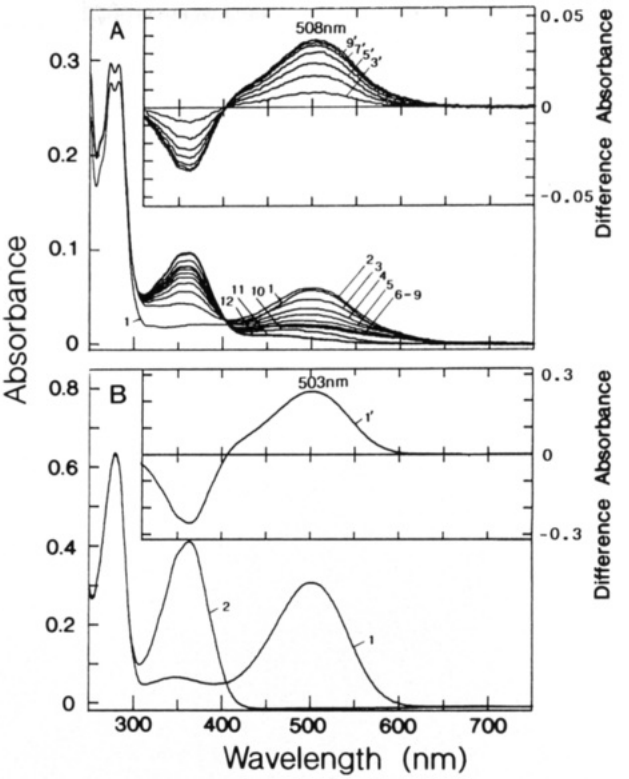

FIGURE 7: Spectral separation of chicken green from rhodopsin by utilizing different susceptibilities against hydroxylamine. (A) Curve 1: Absolute absorption spectrum of the chicken green enriched fraction (the shaded part of the first peak in Figure 6). Immediately after adding 4 μL of 1 M hydroxylamine (pH 6.6) to 0.4 mL of the sample at 4 °C, it was brought to 23 °C (curve 2), and then the spectrum was recorded every hour until the spectrum did not change (curves 3–9). Difference spectra before and after the incubation with hydroxylamine are shown in the inset (curve n' = curves 2 – curve n, n = 3, 5, 7, 9). All the difference spectra showed absorption maxima at 508 nm and formed an isosbestic point at 404 nm. These observations indicate that only chicken green was bleached by the incubation with 10 mM hydroxylamine at 23 °C for 7 h. Then, the pigments still remaining were successively bleached by deep red light (>660 nm, for 80 min; curve 10), red light (>590 nm, for 40 min; curve 11), and yellow light (>480 nm, for 10 min; curve 12). (B) Curve 1: Absolute absorption spectrum of purified rhodopsin fraction (the shaded portion of the second peak in Figure 6). This fraction was mixed with hydroxylamine in the final concentration of 10 mM and then incubated at 23 °C for 7 h, resulting in a negligible spectral change less than 4% (not shown). Irradiation of the sample after the incubation with deep red light (>660 nm, for 80 min) induced no spectral change (not shown). Curve 2: After complete bleaching by irradiation with red light (>590 nm, for 40 min). Further irradiation with orange (>520 nm, for 20 min) or yellow light (>480 nm, for 10 min) also caused no spectral change. Inset: Difference spectrum between curve 1 and curve 2.

al., 1955), only chicken green was progressively bleached by the incubation, as shown in Figure 7A (curves 2–9). The absorption maximum of the difference spectrum between chicken green and its retinal oxime was located at 508 nm (Figure 7A, inset). After complete decomposition of chicken green (74%: curves 2–9) in the fraction in the dark, the compositions of hydroxylamine-resistant pigments remaining were estimated by the partial bleaching to be composed of 12% of chicken red (curves 9 – 10) and 14% of rhodopsin (curves 10 – 11). The yield of chicken green (the shaded part of the first peak in Figure 6) was 0.92 ΔOD·mL, corresponding to 60% recovery of the chicken green applied to the column. In the tailing part, 0.12 ΔOD·mL (8%) chicken green was recovered. Thus, the total recovery of chicken green was calculated to be 68%. The absolute absorption spectrum of the chicken green enriched fraction (Figure 7A) displayed two split

peaks in the UV region. The peak located at about 270 nm is probably not originated from an intrinsic absorption of the protein (at about 280 nm), but from CHAPS and/or PC in the buffer, because it was sometimes observed when a sample in CHAPS–PC buffer was concentrated.

(*ii*) *Rhodopsin.* The second peak fraction (the shaded part in Figure 6) was eluted by increasing concentration of NaCl to 100 mM dissolved in buffer D-20. This fraction contained 10.2 ΔOD·mL rhodopsin corresponding to 69% of the rhodopsin applied to the column. The ratio between absorbances at 280 nm and at 503 nm was 2.0 (Figure 7B). This fraction was subjected to the SDS–PAGE analysis (Figure 5, lane Rh). The molecular mass of rhodopsin was estimated to be 37 kDa in the gel.

Discussion

Using purified or partially purified preparations of cone pigments in CHAPS–PC, we have estimated the absorption maxima of four kinds of cone pigments by difference spectral measurements. The difference absorption maximum of chicken red was evaluated to be 571 nm (Figure 4), which is slightly red-shifted from the value (562 nm) reported in digitonin solution (Wald et al., 1955; Matsumoto & Yoshizawa, 1982), while the maximum of rhodopsin in CHAPS–PC (503 nm) was close to that in digitonin (500 nm). Since the chromophore binding site of chicken red is supposed to be exposed to the surface of the molecule more than that of rhodopsin (Matsumoto et al., 1975), the spectral properties depending on changes of structure of the protein surrounding the chromophore may be affected by the detergent more easily in chicken red than rhodopsin. In fact, we found that chicken red in CHAPS decomposed even in the dark and lost its regenerability in the absence of lipids. Since the absorption maximum of chicken red in the CHAPS–PC system (571 nm) is very close to the value in the retina (569 nm; Bowmaker & Knowles, 1977) or in membranes (572 nm; Fukada & Yoshizawa, 1982), it is suggested that the native conformation of chicken red, or strictly its retinal binding site, is preserved in the CHAPS–PC system better than in digitonin. This is consistent with the notion that the conformation of rhodopsin in CHAPS might be closer to that of the native state than in digitonin, as expected from the analyses of the circular dichroism spectra (Kropf, 1982).

The short-wavelength pigments and chicken red were coeluted from the ConA–Sepharose column with 1.5 mM methyl α-mannoside (fractions 2 and 3 in Figure 3). For the purpose of separation of the short-wavelength pigments from chicken red, the concentration of methyl α-mannoside for the elution was lowered to 0.6 or 1 mM. However, the short-wavelength pigments could not be separated completely from the chicken red, resulting in lower recoveries.

It was usually very hard to measure the difference absorption spectra of the short-wavelength pigments precisely in the CHAPS–PC extract, even if the partial bleaching method was applied, because their concentrations in the extract were much lower than those of chicken red and rhodopsin. Since the initial eluate from the ConA–Sepharose column with 1.5 mM methyl α-mannoside (Figure 3, fraction 2) was enriched with short-wavelength pigments, the absorption maximum of chicken violet (425 nm) could be estimated from the difference spectra (Figure 4A, inset, curve 4′) between chicken violet and its photoproduct, retinal oxime plus V-photopsin (opsin moiety of chicken violet). However, this difference spectrum is distorted owing to the absorption of retinal oxime having an absorption maximum at 363 nm, close to that of chicken violet. In order to evaluate the real absorption

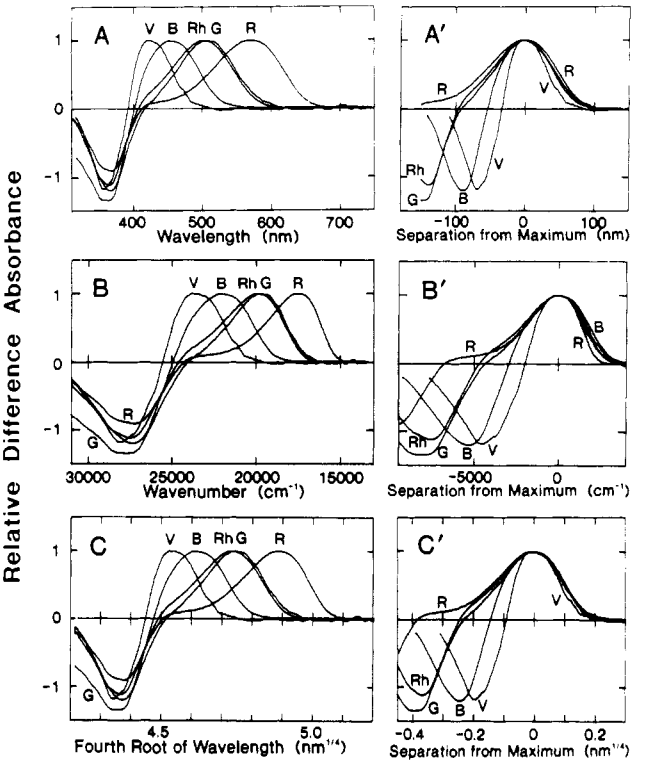

FIGURE 8: Difference absorption spectra between chicken violet (V), blue (B), green (G), red (R), or rhodopsin (Rh) and respective retinal oxime plus opsin plotted on a scale of wavelength (A), wavenumber (B), or fourth root of the wavelength (C). The maximum absorbances were normalized to 1.0. The curves in A, B and C were replotted against the separation from the maxima: $\lambda - \lambda_{max}$ (A′), $1/\lambda - 1/\lambda_{max}$ (B′), and $\lambda^{1/4} - \lambda_{max}^{1/4}$ (C′), respectively. The difference spectrum of chicken green was calculated from the difference spectrum before and after the bleaching by 10 mM hydroxylamine at 4 °C without irradiation, while others were calculated from difference before and after the irradiation at 4 °C.

maximum of chicken violet, the difference spectrum must be corrected as follows. Concerning the similarity of the shapes of absorption spectra of visual pigments, a nomogram using a scale of wavenumber was proposed on the basis of the observation that the absorption spectrum of a visual pigment whose absorption maximum is located at shorter wavelength is narrower than that with a maximum at longer wavelength (Dartnall, 1953). When the half-bandwidths of visual pigments having the same chromophore are calculated on a scale of wavenumber, the values are in good linear relationship to their absorption maxima according to Ebrey and Honig (1977). Using the difference spectra instead of the absolute spectra, we have calculated the half-bandwidths of five kinds of chicken visual pigments and found that particularly those of the short-wavelength pigments deviated from the linear relationship. In fact, the half-bandwidths of chicken blue and violet calculated from the difference spectra (4055 and 3214 cm^{-1}, respectively) were smaller than the values (about 4800 and 5200 cm^{-1}, respectively) predicted by the nomogram of Ebrey and Honig (1977), indicating that the difference spectra were narrowed by the overlapping of absorption of retinal oximes. Thus, the namogram cannot be applied to estimate the absolute absorption maximum of chicken violet. Recently, it was found that the absorption spectra of visual pigments of *Macaca fascicularis* (Barlow, 1982) and of humans (Darnall et al., 1983) plotted on a scale of the fourth root of the wavelength coincided in shape with each other. In fact, the difference spectra between five kinds of chicken visual pigments and their retinal oximes plus opsins were plotted on a scale of wavelength (Figure 8A), wavenumber (B), or fourth root

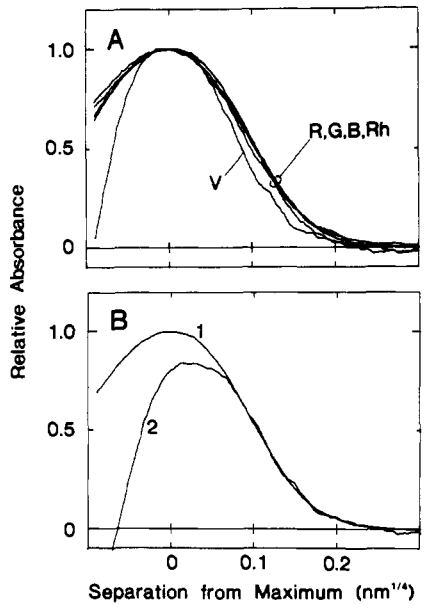

FIGURE 9: Correction of the absorption maximum of chicken violet. (A) Difference spectra between five kinds of chicken visual pigments and their retinal oximes plus opsins were plotted against separation from maxima on a scale of fourth root of the wavelength (a part of Figure 8C′ was enlarged). (B) Curve 1: Averaged difference spectra of four chicken pigments (curves R, G, B, and Rh in pane A). Curve 2: Difference spectrum of chicken violet corrected by a computer simulation. The absorption maximum was estimated at 415 nm. For the simulation of the spectrum of chicken violet, the absorbances recorded at an interval of 1 nm were converted into absorbances against fourth root of the wavelength at an interval of 1×10^{-3} $nm^{1/4}$. Then three parameters were used. $P1$ is the shortest wavelength where retinal oxime has no absorbance. This parameter was varied from 430 to 450 nm at an interval of 1 nm. The absorbance of difference spectrum of chicken violet (curve V in panel A) was multiplied by the second parameter, $P2$, and then shifted to longer wavelength by the last parameter $P3$ (in $nm^{1/4}$) so as to fit with the averaged spectrum at the wavelength ($P1$) on a scale of fourth root of the wavelength. It should be noted that a combination of $P1$ and $P2$ values gives a $P3$ value. The best fitting was estimated by the smallest S/N value, where S denotes a sum of the squares of the difference absorbances at every 10^{-3} $nm^{1/4}$ between the averaged spectrum and the modified spectrum of chicken violet and N denotes the number of squares in the summation. Since chicken violet has no absorbance at 520 nm, the S/N value was calculated in the region from $P1$ (nm) to 520 (nm). Thus the spectrum of chicken violet was corrected by the three parameters $P1$ = 445 nm, $P2$ = 0.84, and $P3$ = 0.027 $nm^{1/4}$ (curve 2), the maximum of which was estimated at 415 nm. Even if $P1$ was changed from 440 to 450 nm, the simulated maximum varied only slightly from 414 to 416 nm.

of the wavelength (C), and their absorption peaks were shifted at a position on each scale (A′, B′, or C′, respectively). On a scale of fourth root of the wavelength the difference spectra of chicken red, green, blue, and rhodopsin except for violet were in fairly good agreement with each other in the longer wavelength region (Figure 8C′). The divergence of chicken violet suggests that the difference absorption maximum of chicken violet should be shifted to longer wavelengths than the real absorption maximum owing to distortion by the absorption of retinal oxime. The spectrum of chicken violet was simulated in a way to fit best (see legend to Figure 9) with the averaged spectrum of the other pigments (chicken red, green, and blue and rhodopsin) in the longer wavelength region where retinal oxime has no absorbance. Thus 415 nm was estimated to be the absorption maximum of chicken violet (Figure 9). This value is in good agreement with those determined from analyses of electroretinograms (415 nm; Norren, 1975) and of early receptor potential (413 nm; Govardovskiĭ & Zueva, 1977). This provides additional evidence that the spectral properties of visual pigments in CHAPS-PC are close to those in vivo.

Through the purification procedures, we have detected four kinds of cone pigments and rhodopsin in chicken retina. The ratio of pigments was roughly estimated from the ΔOD·mL of each pigment in the fractions from the ConA column as follows: rhodopsin:red:green:blue:violet = 49:40:5:5:1. This ratio is not always equal to that of population of corresponding visual cells in a chicken retina due to many factors, for example, decomposition of pigments during the procedures, efficiency of the extraction, differences in the molar extinction, the cell size, concentration of the pigment in the cell, etc. However the content of chicken red in the retina was highest among all the cone pigments. Microspectrophotometric (Bowmaker & Knowles, 1977) and immunohistochemical analyses (Szél et al., 1986; Shichida et al., 1989) showed that both double cones and one or more type(s) of single cones contained chicken red. Accordingly, the high content of chicken red may be ascribed to the double cone population.

The success in purification of chicken red having the native spectral properties enables us to analyze the physiological functions of chicken red in detail. As the first application of a novel CHAPS-PC system, the purified chicken red was reconstituted into phosphatidylcholine liposomes. Then chicken red as well as bovine or chicken rhodopsin, only when irradiated, enhanced the binding of a GTP analogue [guanosine 5′-(β,γ-imidotriphosphate)] to the α-subunit of bovine transducin (Fukada et al., 1989). Thus, purified cone pigments in the CHAPS-PC system are now ready for wide application to the functional analysis of cone photoreceptor cells.

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Fetal Lamb 3β,20α-Hydroxysteroid Oxidoreductase: Dual Activity at the Same Active Site Examined by Affinity Labeling with 16α-(Bromo[2'-^{14}C]acetoxy)progesterone†

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ABSTRACT: 3β,20α-Hydroxysteroid oxidoreductase was purified to homogeneity from fetal lamb erythrocytes. The M_r 35 000 enzyme utilizes NADPH and reduces progesterone to 4-pregnen-20α-ol-3-one [K_m = 30.8 μM and V_{max} = 0.7 nmol min^{-1} (nmol of enzyme)$^{-1}$] and 5α-dihydrotestosterone to 5α-androstane-3β,17β-diol [K_m = 74 μM and V_{max} = 1.3 nmol min^{-1} (nmol of enzyme)$^{-1}$]. 5α-Dihydrotestosterone competitively inhibits (K_i = 102 μM) 20α-reductase activity, suggesting that both substrates may be reduced at the same active site. 16α-(Bromoacetoxy)progesterone competitively inhibits 3β- and 20α-reductase activities and also causes time-dependent and irreversible losses of *both* 3β-reductase and 20α-reductase activities with the same pseudo-first order kinetic $t_{1/2}$ value of 75 min. Progesterone and 5α-dihydrotestosterone protect the enzyme against loss of the two reductase activities presumably by competing with the affinity alkylating steroid for the active site of 3β,20α-hydroxysteroid oxidoreductase. 16α-(Bromo[2'-^{14}C]acetoxy)progesterone radiolabels the active site of 3β,20α-hydroxysteroid oxidoreductase wherein 1 mol of steroid completely inactivates 1 mol of enzyme with complete loss of both reductase activities. Hydrolysis of the ^{14}C-labeled enzyme with 6 N HCl at 110 °C and analysis of the amino acid hydrolysate identified predominantly N^{π}-(carboxy[2'-^{14}C]methyl)histidine [His(π-CM)]. Digestion with trypsin of the ^{14}C-labeled enzyme, isolation of the radioactive peptides, and amino acid sequence analysis showed the modified amino acid to be in the sequence H_2N-Tyr-Val-Ala-Val-Met-Pro-Pro-Pro-Ile-Gly-Asp-His(π-CM)-Pro-Leu-Thr-Gly-Ala-Tyr-Tyr-COOH. The results suggest that (1) 5α-dihydrotestosterone and progesterone are reduced at one and the same active site of 3β,20α-hydroxysteroid oxidoreductase, (2) the ^{14}C-labeled peptides isolated from affinity labeling of 3β,20α-hydroxysteroid oxidoreductase with 16α-(bromo[2'-^{14}C]acetoxy)progesterone form part of the steroid-binding region at the active site, and (3) the D-ring of 16α-(bromo[2'-^{14}C]acetoxy)progesterone most likely proximates a His residue as the steroid binds to and reacts with the catalytically active site of fetal lamb 3β,20α-hydroxysteroid oxidoreductase.

Red blood cells from fetal lambs or calves convert progesterone to 4-pregnen-20α-ol-3-one (Nancarrow & Seamark 1968; Nancarrow et al., 1981a; Sharaf & Sweet, 1982). The daily production of progesterone by the pregnant ewe is about 2.7 mg/day, while 700 mL of fetal lamb's blood can theoretically metabolize more than 18 mg of progesterone/h (Short, 1959; Nancarrow & Seamark, 1968). Thus, the total metabolic capacity of fetal lamb blood exceeds the daily maternal output of progesterone by a factor of 160.

20α-Reductase activity in fetal lamb blood continues to increase throughout pregnancy and then rapidly disappears from the newborn (Nancarrow, 1983). 3β-Reductase activity accompanies 20α-reductase activity in fetal red blood cells from species in the family Bovidae (Nancarrow et al., 1981a,b; Sharaf & Sweet, 1982). To further study the nature of the 3β-reductase and 20α-reductase activities in fetal lamb blood, attempts were made to purify what was earlier believed to be two different enzymes. During isolation of 20α-hydroxysteroid oxidoreductase the enzyme was also found to possess 3β-reductase activity (Chen et al., 1987) like the enzyme isolated from bovine fetal blood (Nancarrow et al., 1981b; Sharaf &

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