Molecular Properties of Chimerical Mutants of Gecko Blue and Bovine Rhodopsin†

Daisuke Kojima,‡ Tomonori Oura,‡ Osamu Hisatomi,‖ Fumio Tokunaga,‖ Yoshitaka Fukada,⊥ Tôru Yoshizawa,# and Yoshinori Shichida*,‡

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan, Department of Earth and Space Science, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan, Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Tokyo 153, Japan, and Department of Information Systems Engineering, Faculty of Engineering, Osaka Sangyo University, Daito, Osaka 574, Japan

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ABSTRACT: In spite of the high similarity in amino acid sequence between rod visual pigment rhodopsin and gecko blue-sensitive pigment (gecko blue), not only the spectral sensitivities but also the thermal decay rates of the meta II- and III-intermediates are noticeably different from one another [Kojima et al. (1995) *Biochemistry 34*, 1096−1106]. In order to identify the protein region(s) that contain(s) key residues being responsible for the functional difference, we constructed six chimerical mutants derived from gecko blue and bovine rhodopsin, with the aid of protein production in a human embryonic kidney cell line (293S). While the absorption maximum of every mutant was located in between gecko blue (466 nm) and bovine rhodopsin (500 nm), a large blue-shift (18 nm) was observed when the helices I−III of rhodopsin were replaced with those of gecko blue. A time-resolved spectroscopic study demonstrated that this replacement also accelerated the decay rate of the meta II-intermediate. The decay of the meta III-intermediate of the mutants became faster as the compartment of gecko blue was increased. Thus, the faster decay of the meta II-intermediate of gecko blue is largely attributed to residues within helices I−III, while the decay of the meta III-intermediate apparently depends on the overall structure of the protein.

The visual transduction process in photoreceptor cells is initiated by photon absorption by a visual pigment, which contains an 11-*cis*-retinal attached to a specific lysine residue (K296) of a protein (opsin) through a protonated Schiff base linkage. According to studies on bovine rhodopsin (Rh),[1] a rod visual pigment, light absorption causes a *cis-trans* isomerization of the retinylidene chromophore (Yoshizawa & Wald, 1963), leading to structural changes in the opsin moiety. The structural changes result in formation of a physiologically active state, the meta II-intermediate (MII), which can bind to and activate a retinal GTP-binding protein, transducin (Fukada & Yoshizawa, 1981; Kühn et al., 1981; Stryer et al., 1981). These processes are well characterized by biochemical and spectroscopic techniques. Several lines of evidence have now suggested that the longer lifetime of the meta II-intermediate of rhodopsin than that of the cone visual pigment is one of the molecular bases for the larger photoresponse of rods than cones (Shichida et al., 1992, 1994; Okada et al., 1994), because the photoresponse amplitude of the cells is related to the extent of amplification of the light signal which depends on how many transducins are activated (Kühn et al., 1981; Stryer et al., 1981). Thus, it is of interest to delineate specific stretches of amino acids which contributed to the characteristic lifetime of MII of Rh.

Gecko blue (GB) is one of the visual pigments in the retina of a nocturnal lizard, Tokay gecko (*Gekko gekko*). Though the amino acid sequence is highly similar (>70%) to those of green-sensitive visual pigments such as vertebrate rhodopsins (Kojima et al., 1992), the decay of MII-GB into *all-trans*-retinal plus opsin, through the next intermediate meta III (MIII), is much faster than that of MII-Rh (Kojima et al., 1995). In addition, the absorption maximum (λ_{max}) of GB at 467 nm in the photoreceptor cells (Crescitelli et al., 1977; Loew, 1994) is blue-shifted by 33 nm from those of vertebrate rhodopsins (about 500 nm). To identify the structural basis for the diversities of the λ_{max} and the decay speeds of MII and MIII between Rh and GB, we constructed several chimerical mutants composed of different parts of GB and Rh. Spectroscopic studies on the mutants revealed specific regions responsible for the functional diversities between these proteins.

MATERIALS AND METHODS

Expression Constructs and Cells. The coding region of bovine Rh cDNA (BRhk), with a Kozak's consensus sequence (CCACC) at the 5′-end, was derived from cDNA clone bd20 (Nathans & Hogness, 1983), while a *Dra*I−

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* To whom correspondence should be addressed.
‡ Kyoto University.
‖ Osaka University.
⊥ The University of Tokyo.
Osaka Sangyo University.

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GB, gecko blue-sensitive visual pigment (gecko blue); GTP, guanosine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; λ_{max}, absorption maximum (maxima); MI, meta I-intermediate; MII, meta II-intermediate; MIII, meta III-intermediate; PC, L-α-phosphatidylcholine from fresh egg yolk; Rh, rhodopsin.

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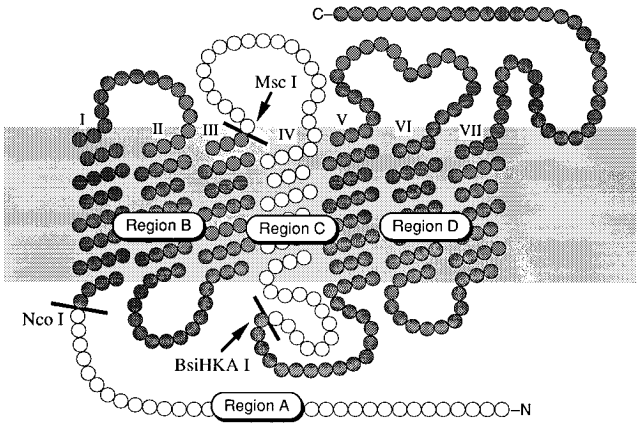

FIGURE 1: Secondary structure model of gecko blue (GB). Three restriction sites (*Nco*I, *Msc*I, and *Bsi*HKAI) were used to combine two DNA fragments encoding GB and bovine rhodopsin (Rh), to produce chimerical mutants. The three positions divide the protein into four fragments, that is, regions A, B, C, and D termed as indicated.

*Eco*O109 I fragment containing the coding region of GB (GZ1DEo) was from cDNA clone GZ1 (Kojima et al., 1992). For each of these DNA fragments, a *Hin*dIII site was attached to the 5′-end, and an *Eco*RI site to the 3′-end. Chimerical DNA fragments were constructed by restriction fragment replacements (Britt et al., 1993) of BRhk and GZ1DEo with three restriction sites (*Nco*I, *Msc*I and *Bsi*HKAI; see Figure 1). Each of the constructed fragments was subcloned into pBluescript II KS+ (Stratagene), and sequenced by the dideoxy termination method with Sequenase (Amersham). Each of the *Hin*dIII−*Eco*RI inserts was recloned into the mammalian expression plasmid pUSRα (Kayada et al., 1995), which is a derivative of pUC-SRα (Shimamoto et al., 1993). Finally, the expression plasmids contained the following regions in a head-to-tail manner: an SRα promoter (Takebe et al., 1988); an SV40 late-gene splice junction; the opsin coding region (described above); and an SV40 late region polyadenylation signal.

A suspension-adapted variant, 293S (Nathans et al., 1989), of the human embryonic kidney cell line (ATCC CRL 1573) was used as the recipient for transient transfection. The cells were grown in 10% fetal calf serum, 50:50 DMEM/F12 with low glucose (Gibco) at 37 °C in a 5% CO_2 atmosphere. In a typical experiment, 200 μg of the opsin expression plasmid and 10 μg of pRSV-TAg (an SV40 T-antigen expression plasmid) were coprecipitated onto 20 9-cm diameter plates of 293S cells by the calcium phosphate method with a glycerol shock (Gorman et al., 1990). The cells were collected 60−70 h after transfection with phosphate-buffered saline (PBS) containing 5 mM EDTA, and stored at −80 °C until use.

Membrane Preparation. The following procedures were performed at 4 °C, according to the method of Nathans (1990) with some modifications. The collected cells were homogenized with 8.6% (w/v) sucrose in buffer Pm (50 mM HEPES−NaOH, 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), by using an ULTRA-TURRAX homogenizer (IKA-WERK), and then layered on top of 40% (w/v) sucrose in buffer Pm, followed by centrifugation in a swing-bucket rotor (SRP28-SA) at 110000*g* for 30 min. The membranes (3−5 mL) floating at the interface between 8.6% and 40% sucrose layers were collected.

Reconstitution with 11-cis-Retinal and Extraction of Proteins. The following manipulations were performed at 4 °C in the dark or under dim-red light (>660 nm). The membrane preparation containing expressed opsin (described above) was mixed with an excessive amount (about 10 nmol) of 11-*cis*-retinal (in ethanol), purified by means of HPLC (Maeda et al., 1978), and incubated for 12−24 h. To remove excess 11-*cis*-retinal (less than 10 μM) from the sample, a 1 M solution of neutralized hydroxylamine was added to the sample to give the final concentration of 10 mM, followed by washing twice with 4% (w/v) BSA in buffer Pm and once with buffer Pm. After the washing procedure, the precipitated membranes were suspended with 400 μL of buffer E (0.75% CHAPS, 1 mg/mL PC, 50 mM HEPES−NaOH, 140 mM NaCl, 1 mM DTT, 1 μg/mL aprotinin, and 1 μg/mL leupeptin, pH 6.5 at 4 °C), homogenized with a micro-homogenizer, and incubated for 30 min. The solubilized proteins were isolated from insoluble materials by centrifugation at 110000*g* for 15 min, and mixed with glycerol to give a final concentration of 20% (w/v). It was confirmed that hydroxylamine was completely removed by the washing procedure, because addition of 11-*cis*-retinal to an aliquot of the extract showed no formation of retinal-oxime (a product of retinal plus hydroxylamine).

Preparation of GB from Retina. GB for time-resolved spectroscopy was extracted from retinas of *Gekko gekko* and purified by two steps of column chromatography, as previously reported (Kojima et al., 1995). Since the GB fraction contained a small amount of a green-sensitive visual pigment (gecko green), the sample was irradiated at 5 °C with a red light (>610 nm) to decompose only gecko green, prior to the experiment.

Spectroscopy. Absorption spectra were recorded with a Shimadzu Model MPS-2000 spectrophotometer, from which the data were transferred to an NEC PC-9801RA personal computer. The sample in the optical cell (light pass, 1 cm; width, 2 mm) was kept at the required temperature by using a thermostated cell holder equipped in the spectrophotometer. A 1-kW tungsten−halogen lamp (Rikagaku Co. Ltd.) was used as a light source for irradiation of the sample. Wavelengths of the irradiation light were selected by a cutoff filter [VY52 (transmitting light >500 nm), VO55 (>530 nm), VO56 (>540 nm), VO58 (>560 nm), or VR63 (>610 nm); Toshiba Co. Ltd.]. To remove heat from the irradiation light, a water layer with 5-cm width was placed between the light source and the filter. Some of the collected data were subjected to calculations (smoothing, normalization, fitting, or Fourier transform) using a Macintosh Quadra 650 equipped with Igor software.

RESULTS

Expression of GB and GB/Rh Chimerical Mutants in 293S Cells. The coding region (GZ1DEo) of cDNA clone GZ1 obtained from a gecko retinal cDNA library (Kojima et al., 1992) was cloned into the mammalian expression vector pUSRα, and transfected into a human embryonic kidney cell line, 293S. The absorption maximum (λ_{max}) of the expressed protein reconstituted with 11-*cis*-retinal was located at 466 nm (Figure 2, panel A), which is in good agreement with those of native GB in the photoreceptor cell [467 nm (Crescitelli et al., 1977; Loew, 1994)] and in the purified form [465 nm (Kojima et al., 1995)].

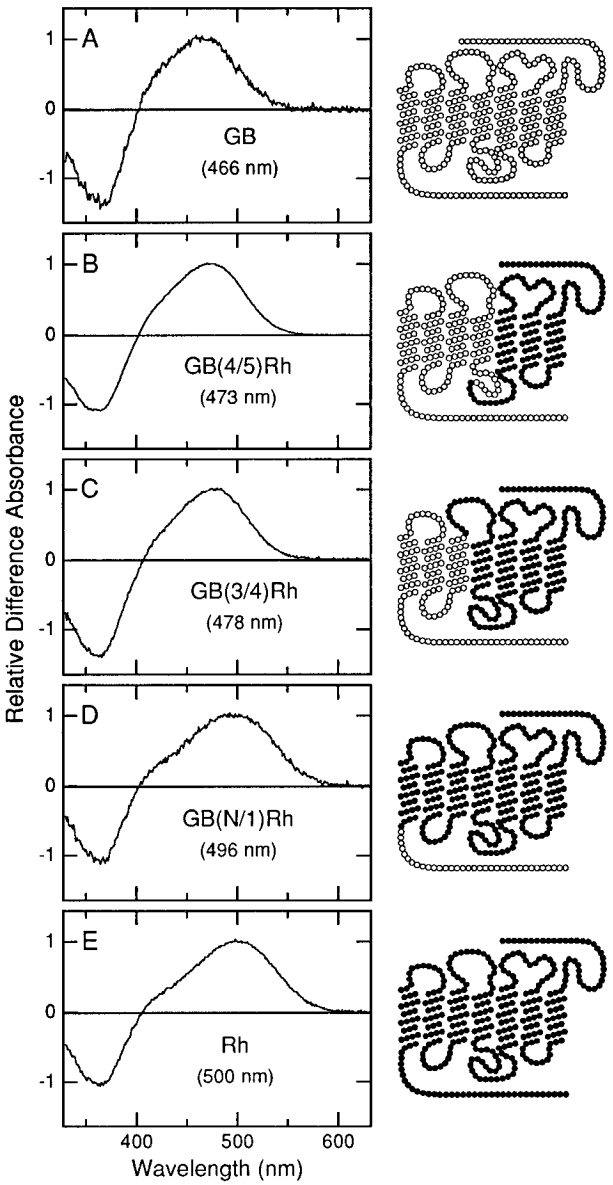

FIGURE 2: Absorption spectra of GB, Rh, and their chimerical mutants expressed in 293S cells. GB (panel A), GB(4/5)Rh (panel B), GB(3/4)Rh (panel C), GB(N/1)Rh (panel D), and Rh (panel E) were transiently expressed in 293S cells. The expressed protein was extracted with a detergent (CHAPS), reconstituted with 11-*cis*-retinal, and irradiated with a yellow light (>500 nm) in the presence of 10 mM hydroxylamine. Each panel shows the difference spectrum obtained from the spectral measurements before and after irradiation. The wavelength at the positive peak (λ_{max}) of each spectrum is indicated in parentheses. All the difference absorbances at the λ_{max} were normalized to 1.0. The original values of difference absorbances at the λ_{max} were 0.004 (panel A), 0.05 (panel B), 0.006 (panel C), 0.004 (panel D), and 0.018 (panel E). Models of the chimerical mutants are shown in the right-hand sides of these panels, where open and closed circles indicate the residues of GB and Rh, respectively.

Chimerical cDNAs composed of GB and Rh were constructed from combinations of their respective cDNA fragments, GZ1DEo and BRhk. Restriction fragment replacements at an *Nco*I site (Figure 1) produced two chimerical mutants, GB(N/1)Rh[2] and Rh(N/1)GB. The protein encoded by the former consists of the N-terminal fragment (region A; see Figure 1) of GB and the rest (regions B, C, and D) from Rh, while the latter consists of region A of Rh and regions B, C, and D of GB. On the other hand, two chimerical mutants, GB(3/4)Rh and Rh(3/4)GB, were produced by using the *Msc*I site, and two chimerical mutants, GB(4/5)Rh and Rh(4/5)GB, by using the *Bsi*HKAI site.

Each of the cDNAs encoding the mutants was cloned into pUSRα and transfected into 293S cells. All three GB(*x*/*y*)-Rh mutant proteins bound 11-*cis*-retinal (Figure 2), to give their λ_{max} at 473 nm [GB(4/5)Rh], 478 nm [GB(3/4)Rh], and 496 nm [GB(N/1)Rh], while the λ_{max} of expressed GB and Rh were 466 and 500 nm, respectively. The noticeable difference in λ_{max} between GB(3/4)Rh and GB(N/1)Rh (18 nm) indicates that the most responsible residues for the difference between spectral sensitivities of GB and Rh reside within region B (Figure 1).

Of the three Rh(*x*/*y*)GB mutants, only Rh(N/1)GB was expressed in cultured cells and bound 11-*cis*-retinal (data not shown) to give its λ_{max} at 468 nm. The chimerical proteins of Rh(3/4)GB and Rh(4/5)GB were not detected by an antibody to Rh.

Thermal Reaction of Meta-Intermediates. Thermal reactions of photobleaching intermediates after the irradiation of GB(4/5)Rh and GB(3/4)Rh at 5 °C were compared with those of GB and Rh (Figure 3). In Rh, a decrease of the absorbance at 380 nm and an increase at 450 nm were observed (Figure 3D). This spectral change reflects the thermal conversion of the meta II-intermediate (MII) into the meta III-intermediate (MIII). A similar study on GB (the native pigment from gecko retina) showed only the decay process of MIII-GB into *all-trans*-retinal plus opsin (Figure 3A), indicating that MII-GB decays much faster than MII-Rh, as shown in a previous paper (Kojima et al., 1995). Because only an extraordinarily small amount of expressed GB was available, we were unable to measure the spectral change for the MIII decay of the expressed GB at 5 °C. However, the spectral change observed at −14 °C is similar in shape to that observed in the native GB (Figure 4), and the kinetic measurement at 460 nm at 5 °C showed that the decay process of MIII seemed to be completed within 5 min (data not shown). Therefore, the expressed GB shows a bleaching process similar to that of the native GB, although the intermediate is somewhat unstable as compared with that of the native GB.

The spectral changes of the mutant GB(4/5)Rh, showing a decrease of absorbance at 460 nm and a concurrent increase at 380 nm (Figure 3B), were similar to those of GB. However, the time constant calculated from the change in absorbance at 460 nm was 900 min, which is much larger than that of the native GB (30 min) or the expressed GB (<5 min).

As for GB(3/4)Rh, little spectral change was observed up to 320 min at 5°C after the irradiation (Figure 3C). This was ascribed to the high stability of MIII-GB(3/4)Rh, because the subsequent incubation beyond 320 min at 5 °C induced spectral changes, which seemed to represent the decay of MIII (data not shown). The decay of MIII was clearly observed when the sample was irradiated at a higher temperature (15 °C), exhibiting spectral changes (Figure 3C, inset) similar to those observed in GB(4/5)Rh at 5 °C (Figure

[2] The chimerical mutant was termed GB(N/1)Rh, since its N-terminal segment was derived from GB and the segment from helix I to the C-terminus was derived from Rh (see Figure 1). The other mutants were termed in a similar manner; for example, Rh(3/4)GB was composed of the N-terminus to helix III of Rh and the helix IV to the C-terminus of GB.

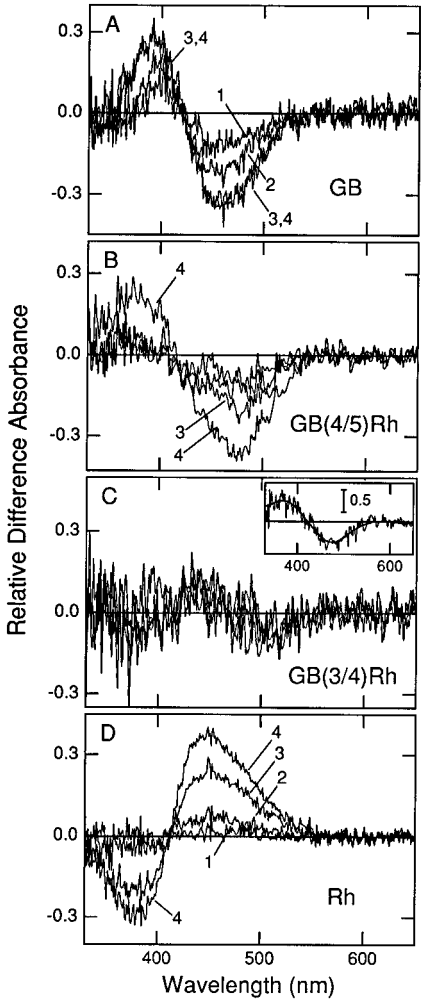

FIGURE 3: Thermal reaction of meta-intermediates of GB, Rh, and their chimerical mutants. GB(4/5)Rh (panel B), GB(3/4)Rh (panel C), and Rh (panel D) were transiently expressed in 293S cells. GB (panel A) was extracted and purified from gecko retinas. Each sample was cooled to 5 °C and irradiated for 30 s with orange light [>530 nm for GB and GB(4/5)Rh, >540 nm for GB(3/4)Rh, and >560 nm for Rh]. The absorption spectra of the irradiated sample were measured repeatedly at a constant temperature (5 °C) in the dark. Curves 1−4 were obtained by subtracting the spectrum immediately after irradiation from the spectra measured at 5 min (curve 1), 20 min (curve 2), 80 min (curve 3), and 320 min (curve 4) after irradiation. The spectra in each of panels B and C were the means of spectra from the two independent experiments, and treated with an operation of smoothing. The inset of panel C shows a difference spectrum in a similar experiment of GB(3/4)Rh at 15 °C, which was obtained by subtracting the spectrum measured immediately after irradiation from those at 320 min after the irradiation. The smoothed line in the inset was obtained with a noise-reduction method, where the noises having higher frequencies were removed by using a Fourier transform operation. All the spectra were normalized so that 1.0 absorbance unit of the photopigment should be photoreacted in each experiment. The original amounts (absorbance units) of photoreacted pigments were 0.013 (panel A), 0.008 (panel B), 0.005 (panel C), and 0.021 (panel D).

3B). Thus, we concluded that MIII-GB(3/4)Rh forms faster than MIII-Rh, but decays slower than MIII-GB(4/5)Rh. Furthermore, our preliminary experiments at 15 °C indicated that the difference in MIII decay rate between GB and GB-(4/5)Rh is larger than those among GB(4/5)Rh, GB(3/4)Rh, and Rh (Kojima et al., unpublished data), suggesting that the decay process of MIII heavily depends on the type of region D (Figure 1).

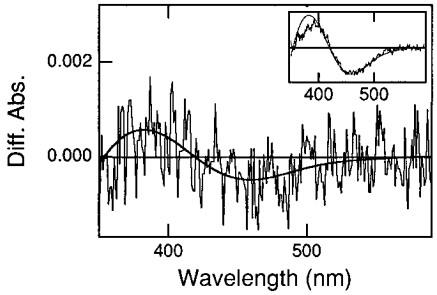

FIGURE 4: MIII decay of expressed GB at −14 °C. GB was transiently expressed in 293S cells. The sample was cooled to −14 °C and irradiated for 30 s with orange light (>530 nm). The sample was kept in the dark at −14 °C. The spectrum shown here is the difference between the one measured 80 min after irradiation and the one immediately after irradiation. From this spectrum, the smoothed curve was obtained with the noise-reduction method described in the legend of Figure 3. In the inset, the smoothed curve is compared with a spectrum of the conversion from MIII into *all-trans*-retinal plus opsin in native GB (curve 3 in Figure 3A). These two curves in the inset were normalized at the negative peak absorbance.

Kinetic experiments of GB(4/5)Rh and GB(3/4)Rh at −14 °C showed increases in absorbance at 380 nm due to the formation of MII. The time constants of the process were about 200 s for GB(4/5)Rh and 700 s for GB(3/4)Rh, which are similar to that observed in native GB (about 300 s). In another kinetic experiment of GB(3/4)Rh at 5 °C, a small increase of the absorbance at 380 nm (MII formation) was followed by a decrease of absorbance. The latter absorbance change is due to the conversion of MII to MIII, and its time constant was estimated to be 200 s, which is also similar to that observed in native GB (about 100 s). Although MII decay in GB(4/5)Rh was not measured directly, one can expect its time constant to be located in between 200 and 100 s. On the other hand, the decay time constant of MII decay in Rh was estimated to be 100 min, which is about 30-fold slower than that in GB(3/4)Rh (200 s). Thus, the difference in MII decay rate between Rh and GB was largely attributed to the amino acid residues within region A−B in Figure 1.

DISCUSSION

Amino Acid Residue(s) Responsible for the Difference in λ_{max} between Gecko Blue and Bovine Rhodopsin. On the basis of similarities of the amino acid sequence, vertebrate visual pigments have been classified into four groups which contain respectively red-, green-, blue-, and violet-sensitive visual pigments (Okano et al., 1992). GB and Rh are members of the green-sensitive group (Kojima et al., 1992), but the λ_{max} of GB is blue-shifted by about 30 nm from that of Rh. The present results indicated that the difference in λ_{max} between GB and Rh is mainly due to the diverged amino acid residues in region B (Figure 2), where 33 positions (out of 98) are different between the 2 proteins. It has been already reported that a double mutation of Rh at positions 83 (Asp → Asn, i.e., D83N) and 122 (Glu → Gln, i.e., E122Q) induces a significant blue-shift of the λ_{max}, up to 20 nm (Zhukovsky & Oprian, 1989) or 25 nm (Fahmy et al., 1993), while a single mutation at each position gives a blue-shift of 0−8.5 nm for D83N or 16−20 nm for E122Q (Sakmar et al., 1989; Zhukovsky & Oprian, 1989; Nathans, 1990; Janssen et al., 1990; Nakayama & Khorana, 1991;

Fahmy et al., 1993). Both sites locate in the region B, and Rh has D83 and E122, while GB has N83 and Q122. Thus, our results are consistent with those reported. Interestingly, the shift in the double mutation (20 or 25 nm in D83N/E122Q) exceeds the difference in λ_{max} between GB(N/1)Rh and GB(3/4)Rh (18 nm), suggesting the presence of additional amino acid residue(s) which shift(s) the λ_{max} to the opposite direction (longer wavelengths). Since it is generally accepted that the spectral shift of the visual pigment originates from the presence of hydroxyl or polarizable residue in the vicinity of the chromophore (Neitz et al., 1991; Kojima et al., 1992; Merbs & Nathans, 1993; Asenjo et al., 1994), the amino acid residues at 10 positions among the candidate 33 positions (described above), in addition to positions 83 and 122, could be responsible for the difference in λ_{max} between GB and Rh.

Thermal Stability of Meta II- and III-Intermediates. The time-resolved spectroscopy identified protein regions which strongly affect the decay processes of MII and MIII. The kinetic measurements showed that the MII decay of GB(3/4)Rh is 30-fold faster than that of Rh, while it is similar to that of GB. Thus, the difference in MII decay rate between GB and Rh is largely attributed to region A−B (Figure 1), that is, helices I−III. On the other hand, MIII decays more slowly as the composition of the region derived from Rh is increased (Figure 3), suggesting that the MIII decay depends on the overall structure of the protein. However, this suggestion should be confirmed by the further investigation using Rh mutants in each of which a single helix is replaced with that of GB.

In the region being predicted to regulate MII decay (region A−B in Figure 1), we should note again the amino acid residues at positions 83 and 122, where Rh has chargeable residues (D83 and E122) and GB has nonchargeable ones (N83 and Q122). In the MII formation process of Rh, changes in the hydrogen-bonding state of D83 and E122 were observed (Fahmy et al., 1993; Rath et al., 1993), and the signals due to the changes were significantly reduced during MII−MIII conversion (Klinger & Brainman, 1992). Furthermore, these residues affect the pH-dependent equilibrium between MI (the preceding intermediate of MII) and MII; D83 shifts the equilibrium toward MI while E122 shifts it toward MII (Weitz & Nathans, 1993). Thus, these two residues are candidates responsible for the difference in MII decay rate between GB and Rh.

Our data suggest that an overall conformational change takes place during MIII decay. Recent studies have indicated that the thermal behavior of the later intermediates is regulated by the electric properties of the visual pigments (Shichida et al., 1994; Imai et al., 1995). Thus, possible residues to regulate the MIII decay rate are the ones inducing the difference in "net charge" between GB and Rh. GB is predicted to be positively charged at neutral pH (Kojima et al., 1992), in contrast to negatively charged rhodopsin (Okano et al., 1992). The difference in net charge between GB and Rh originates from the replacement of chargeable residues (positive → neutral, or neutral → negative) at 10 positions, which are located at regions B, C, and D. Future investigations will test our hypothesis that these positions regulate MIII decay.

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