Presence of Two Rhodopsin Intermediates Responsible for Transducin Activation†

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ABSTRACT: To identify how many rhodopsin intermediates interact with retinal G-protein transducin, the photobleaching process of chicken rhodopsin has been investigated in the presence or absence of transducin by means of time-resolved low-temperature spectroscopy. Singular value decomposition (SVD) analysis of the spectral data showed that a new intermediate called meta I_b is present between formally identified metarhodopsin I (now referred to as meta I_a) and metarhodopsin II (meta II). Since the absorption maximum of meta I_b (460 nm) is similar to that of meta I_a (480 nm), but considerably different from that of meta II (380 nm), meta I_b should have a protonated retinylidene Schiff base as its chromophore. Whereas transducin showed no effect on the conversion process between lumirhodopsin (lumi) and meta I_a, it affected the process between meta I_a and meta I_b and that between meta I_b and meta II. These results suggest that at least two intermediates (meta I_b and meta II) interact with transducin. The addition of GTPγS had no effect on the meta I_b−transducin interaction, while it abolished the ability of transducin to interact with meta II. Thus, meta I_b only binds to transducin, while meta II catalyzes a GDP−GTP exchange in transducin. These results suggest that deprotonation of the Schiff base chromophore is not necessary for the binding to transducin, while changes in protein structure including Schiff base deprotonation are needed to induce the GDP−GTP exchange in transducin.

The visual transduction process in rod photoreceptor cells begins with photon absorption by the visual pigment rhodopsin, which is a member of the family of G-protein-coupled receptors and contains 11-*cis*-retinal as a light absorbing chromophore (Wald, 1968; Hargrave & McDowell, 1992). Light initiates cis-trans isomerization of the chromophore (Hubbard & Kropf, 1958; Yoshizawa & Wald, 1963) to form the primary intermediate, photorhodopsin (Shichida et al., 1984). Subsequent thermal reactions result in formation of several intermediates, each of which has a specific absorption spectrum (Hubbard et al., 1959; Yoshizawa & Wald, 1963; Matthews et al., 1963; Imai et al., 1994), and finally lead to decomposition into *all-trans*-retinal and the protein moiety opsin. During the process, retinal G-protein transducin is activated through a GDP−GTP exchange reaction catalyzed by rhodopsin intermediate(s). Since the interaction between rhodopsin intermediates and transducin is the critical step of the visual transduction cascade, the molecular mechanism leading to the binding and activation of transducin has been highlighted for many years.

The first implication that metarhodopsin II (meta II) could be an active state of rhodopsin came from the biochemical evidence that phosphodiesterase was activated even at low temperature where meta II did not convert to the subsequent intermediate, metarhodopsin III (meta III) (Fukada & Yoshizawa, 1981). Then, spectroscopic measurements indicated that a large amount of meta II was accumulated in the presence of transducin and the accumulation was abolished by guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS)[1] (Emeis et al., 1982; Hofmann, 1985). Thus, it has been generally thought that meta II forms a complex with transducin, and catalyzes GDP−GTP exchange in the α subunit of transducin (Bennett et al., 1982; Kibelbek et al., 1991). These findings also suggested that the deprotonation of the Schiff base, characteristic of meta II, was essential for the activation of transducin (Longstaff et al., 1986; Robinson et al., 1992). However, recent mutagenesis studies suggested little relationship between activation of transducin and Schiff base deprotonation, because transducin was activated by the product whose retinylidene Schiff base was still protonated (Zvyaga et al., 1994). Furthermore, there is no evidence that invertebrate visual pigments activate retinal G-protein via an intermediate having a deprotonated Schiff base chromophore (Suzuki et al., 1995). Thus, it is an intriguing

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; ROS, rod outer segment(s); Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

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argument that molecular events beside the Schiff base deprotonation should be crucial for the activation of transducin by rhodopsin.

Although the photobleaching process of rhodopsin has been extensively investigated by means of various spectroscopic techniques, the interaction with transducin has been investigated only at the meta II stage. Thus, we have investigated the photobleaching process of rhodopsin in the presence or absence of transducin by means of time-resolved low-temperature spectroscopy, to examine whether or not meta II is the sole intermediate which interacts with transducin. Current findings indicate that the intermediate formed before meta II also binds to transducin, though it does not induce the GDP−GTP exchange reaction in transducin. The molecular mechanism leading to the activation of transducin by rhodopsin intermediates is discussed.

MATERIALS AND METHODS

Preparation of Rhodopsin. Rhodopsin was extracted from chicken retinas by a mixture of CHAPS and PC and purified by means of column chromatography (Okano et al., 1989; Imai et al., 1994). The purified rhodopsin in buffer A [0.6% (w/v) CHAPS, 0.8 mg/mL PC, 20% (w/v) glycerol, 50 mM HEPES, 140 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, 50 KIU/mL aprotinin, pH 7.5 at 4 °C] was stored at −80 °C until use. The concentration of rhodopsin in the sample was estimated by its absorbance at the maximum (503 nm, molar extinction coefficient = 40 500; Okano et al., 1992).

Preparation of Transducin. Transducin was purified from fresh bovine retinas according to the method reported by Fukada et al. (1994). Briefly, transducin was extracted from bovine ROS membranes with hypotonic buffer B (5 mM Tris, 0.5 mM $MgCl_2$, 0.1 mM PMSF, 4 μg/mL leupeptin, 50 KIU/mL aprotinin, 1 mM DTT, pH 7.2 at 4 °C) supplemented with 100 μM GTP. Then it was applied to a Blue Sepharose (Pharmacia) column which had been equilibrated with buffer B. Transducin α subunit (Tα) bound to the column, while its $\beta\gamma$ subunit (T$\beta\gamma$) flowed through the column. Thus, Tα was eluted from the column with buffer C (10 mM MOPS, 600 mM NaCl, 2 mM $MgCl_2$, 0.1 mM PMSF, 4 μg/mL leupeptin, 50 KIU/mL aprotinin, 1 mM DTT, pH 7.5 at 4 °C). The eluted fractions containing T$\beta\gamma$ were applied to a DEAE-Toyopearl 650S (Tosoh) column from which T$\beta\gamma$ was eluted with buffer C. Tα and T$\beta\gamma$ fractions were then applied to a gel filtration column, Superdex 75-pg HiLoad 26/60 (Pharmacia), from which they were eluted with buffer C. Tα was eluted as a single peak, while T$\beta\gamma$ was eluted as two peaks, which contained farnesylated/nonmethylated and farnesylated/methylated forms of γ subunits, respectively. It is known that the latter form of Tγ is dominant *in vivo*, and we therefore used the latter fractions for the experiments. The fractions contained >95% of methylated Tγ , which was estimated by the method previously described (Ohguro et al., 1991). Then equal amounts of Tα and T$\beta\gamma$ were mixed, and the buffer was exchanged to 50 mM HEPES, 140 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 4 μg/mL leupeptin, 50 KIU/mL aprotinin (pH 7.5 at 4 °C) using a gel filtration column (PD10, Pharmacia Biotech), followed by concentration to about 150 μM by an ultrafiltration membrane (Centricon-30, Amicon). For the experiments to investigate the effect of Tα or T$\beta\gamma$ on the thermal reactions of rhodopsin intermediates, each subunit (Tα or T$\beta\gamma$) was separately concentrated after changing the buffer. It should be noted that Tα prepared under our experimental conditions is a GDP-bound form (Kleuss et al., 1987; Yamazaki et al., 1987; Noel et al., 1993; Lambright et al., 1994) and can form heterotrimer with T$\beta\gamma$ (Fukada et al., 1994). The protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin as a standard. The sample was stored at −80 °C until use.

Time-Resolved Low-Temperature Spectroscopy. To investigate the effects of transducin and GTPγS on the bleaching process of rhodopsin, the following three samples in buffer A supplemented with 50% glycerol were prepared: Sample A contained 3.01 μM rhodopsin, sample B contained 3.01 μM rhodopsin and 9.60 μM transducin, and sample C contained 3.01 μM rhodopsin, 9.60 μM transducin, and 421 μM GTPγS.

Absorption spectra were recorded on a Shimadzu MPS-2000 recording spectrophotometer interfaced to a personal computer (NEC PC9801RA). An optical cryostat (Oxford, CF1204) with an optical cell of 1-cm light path was used to record the spectral changes at low temperatures. The sample temperature was regulated to within 0.1 °C by a temperature controller (Oxford, ITC4) 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 (Toshiba). A 5-cm water layer was placed in front of the light source to remove heat from the irradiation light. Thermal reactions of intermediates initiated by irradiation of the rhodopsin sample at −35 or −25 °C were monitored by recording absorption spectra with intervals of 2.5−30 min until the reactions were almost saturated. The recording of each absorption spectrum in the wavelength region from 750 to 330 nm required 66 s.

The amount of rhodopsin photoconverted to the intermediate(s) by the irradiation at low temperature (−35 or −25 °C) was estimated as follows: The irradiated sample at −35/−25 °C was warmed to 20 °C, and 1 M hydroxylamine was added to the sample at a final concentration of 10 mM, followed by incubation at this temperature until intermediates produced by the irradiation were completely converted to retinal oxime and opsin. Then the sample was cooled to −35/−25 °C to record the spectrum. To bleach the residual rhodopsin and a small amount of isorhodopsin present in the irradiated sample, the sample was irradiated with yellow light (>500 nm light) at 0 °C, and the spectrum was recorded at −35/−25 °C. The difference spectrum between these spectra at wavelengths longer than 500 nm was then simulated with the separately obtained spectra of rhodopsin and isorhodopsin (Imai et al., 1994). The total amount of intermediate(s) produced was calculated by subtracting the amount of residual rhodopsin and isorhodopsin from that of the original rhodopsin. Under our experimental conditions, about 40% of rhodopsin in the sample was photoconverted to the intermediate(s).

Spectral Analyses. The spectral changes due to the thermal reactions of intermediates were analyzed by SVD (singular value decomposition) and global exponential fitting with computer programs originally written on a NEC PC9821 V10 computer, according to the theory reported previously (Golub & Reinsch, 1970; Hug et al., 1990; Henry & Hofrichter, 1992; Thorgeirsson et al., 1993). First, a set of difference

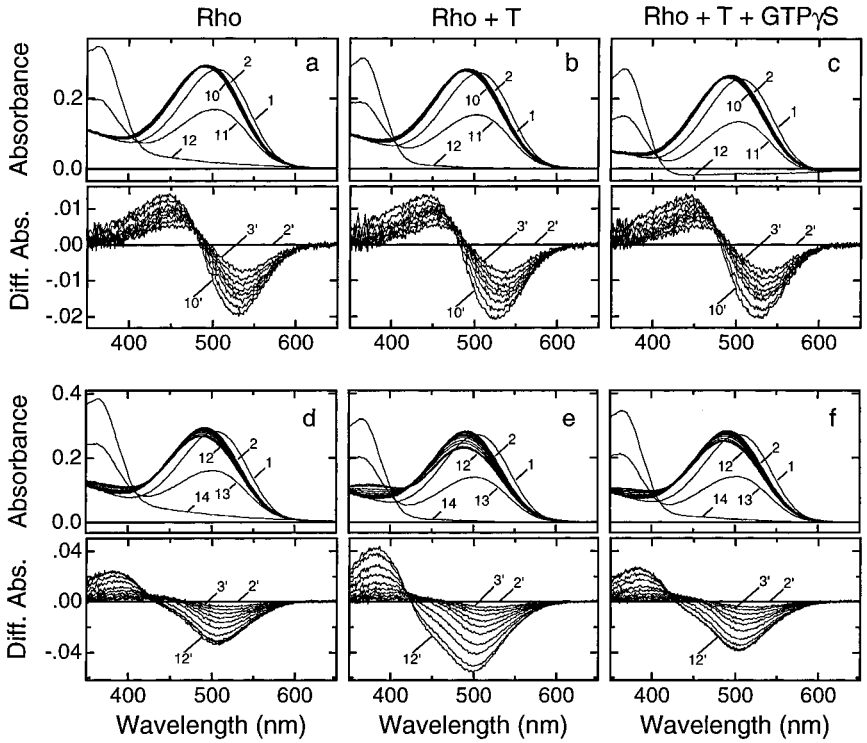

FIGURE 1: Effects of transducin and/or GTPγS on the thermal reactions of rhodopsin intermediates at various temperatures. (a−c) Three samples, each of which contained rhodopsin (sample A) (a), rhodopsin + transducin (sample B) (b), or rhodopsin + transducin + GTPγS (sample C) (c), were cooled to −35 °C (curves 1) and irradiated with >570-nm light for 30 s (curves 2), followed by incubation at this temperature for 2.5, 5, 10, 20, 40, 80, 160, and 320 min (curves 3−10). Then the samples were warmed to 20 °C, and 1 M hydroxylamine was added to the sample at a final concentration of 10 mM. After the spectra were recorded at −35 °C (curves 11), they were irradiated with >500-nm light at 0 °C, and the spectra were measured at −35 °C. (d−f) Three samples, each of which contained rhodopsin (sample A) (d), rhodopsin + transducin (sample B) (e), or rhodopsin + transducin + GTPγS (sample C) (f), were cooled to −25 °C (curves 1) and irradiated with >570-nm light for 30 s (curves 2), followed by incubation at this temperature for 2.5, 5, 10, 20, 40, 80, 160, 320, 640, and 1280 min (curves 3−12). Then the samples were warmed to 20 °C, and 1 M hydroxylamine was added to the sample at a final concentration of 10 mM. After the spectra were recorded at −25 °C (curves 13), they were irradiated with >500-nm light at 0 °C, and the spectra were measured at −25 °C (curves 14). In the lower panel of each figures, difference spectra obtained by subtracting the spectrum recorded immediately after the irradiation from the spectra recorded at later times after the irradiation are presented. Each spectrum is the sum of two spectra recorded by independent experiments.

spectra reflecting the spectral change were calculated by subtracting the spectrum recorded 2.5 min after the irradiation from those recorded at later times after the irradiation. Then the difference spectra whose recording times after the irradiation were near $2.5 \times 2^{0.3i}$ min ($i = 1, 2, ..., n$) were selected from the set of difference spectra. After the number of wavelength points (421 points) of the spectra was reduced to 84 points by averaging over each 5 points, these spectra were subjected to the spectral analysis.

To estimate how many spectrally distinct intermediates are needed to reproduce the spectral change within the noise of the experiments, the difference spectra expressed by a 84 $\times$ n matrix were transferred to a product of three matrices, **U**, **S**, and $\mathbf{V^T}$, by means of SVD. **U** is an 84 $\times$ n matrix whose columns are called basis spectra. **S** is an $n \times n$ diagonal matrix whose diagonal elements are singular values. **V** is an $n \times n$ matrix describing the composition of the original matrix in terms of the basis spectra. Then the b-spectra representing spectral changes and their apparent time constants were calculated after global exponential fitting of the rows of $\mathbf{V^T}$ with exponential time functions. The number of exponential time functions was estimated by the number of meaningful singular values and basis spectra. It should be noted that opposite signed b-spectra are presented in Figures 2, 3, 4, and 5 because of easy comparison with the spectral changes observed by the low-temperature experiments.

RESULTS

In order to investigate which intermediate(s) interact(s) with transducin, we compared the thermal reactions of intermediates of rhodopsin in the sample containing only rhodopsin with those containing rhodopsin and transducin. We also investigated whether or not GTPγS can abolish the coupling between rhodopsin intermediate(s) and transducin. For this purpose, the following three samples were studied; sample A contained only rhodopsin (3.01 μM), sample B contained rhodopsin (3.01 μM) and transducin (9.60 μM), and sample C contained rhodopsin (3.01 μM), transducin (9.60 μM), and GTPγS (421 μM).

Figure 1 shows the absorption spectra of the rhodopsin intermediates in these three samples measured by time-resolved low-temperature spectroscopy. In each experiment, the sample was cooled to −35 or −25 °C and irradiated with >570-nm light for 30 s, followed by recording the spectral changes due to the thermal reactions of intermediates. The spectral changes due to the thermal reactions are expanded in the lower panel of each figure, in which the changes are represented by the difference spectra calculated by subtracting the spectrum recorded immediately after the irradiation from those recorded at later times after the irradiation.

Irradiation of rhodopsin in each sample at −35 °C caused a blue-shift of the absorption spectrum with a slight increase in absorbance, suggesting the formation of a mixture

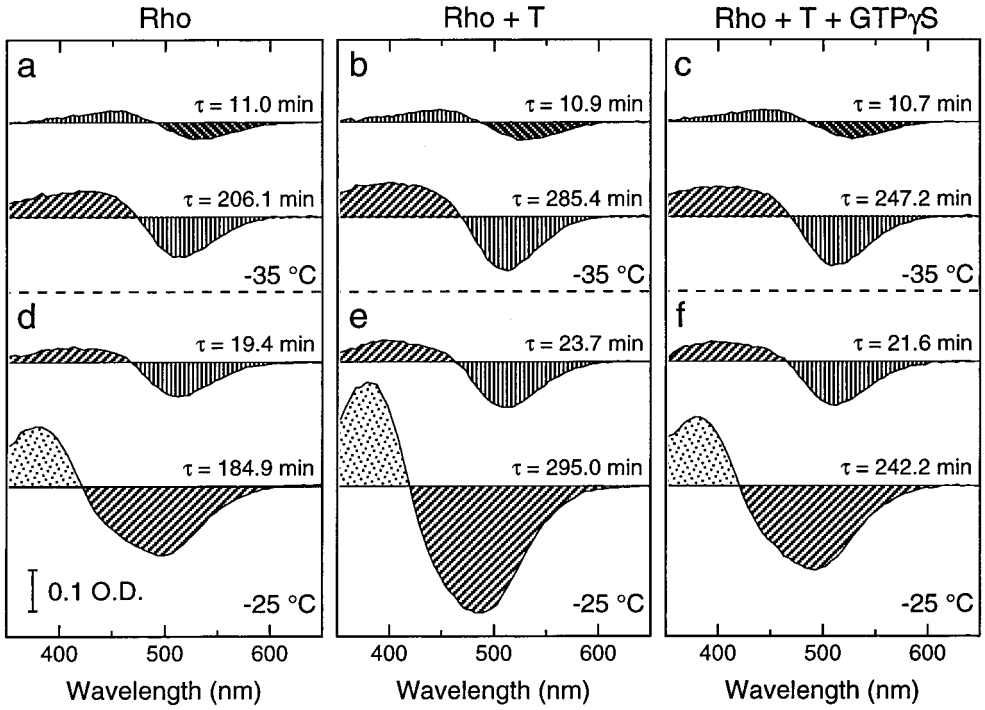

FIGURE 2: B-spectra calculated from the spectral changes observed at −35 and −25 °C using samples containing only rhodopsin (sample A) (a, d), rhodopsin + transducin (sample B) (b, e), and rhodopsin + transducin + GTPγS (sample C) (c, f). In each panel, upper and lower curves are the first and second b-spectra, respectively. Time constants for the respective b-spectra and the measured temperatures are shown in the panels. The b-spectra are normalized so that they represent the changes induced by the photoreaction of 1.0 absorbance of rhodopsin.

containing mainly lumirhodopsin (Imai et al., 1994). Subsequent incubation at this temperature resulted in a decrease in absorbance at about 520 nm and an increase in absorbance at about 440 nm (Figure 1a,b,c). The irradiation at −25 °C also caused a blue-shift of the spectrum with an increase of absorbance, but the shift of maximum was slightly larger than that observed at −35 °C. Subsequent incubation resulted in an increase of absorbance at about 380 nm with a concurrent decrease of absorbance at about 500 nm (Figure 1d,e,f). Although the spectral changes observed at −25 °C represented at least two conversion processes (see below), the final product formed by the incubation should be meta II, because it displayed a large absorbance at about 380 nm. The decay of meta II was not observed at −25 °C under our experimental conditions, while it was observed at −10 °C (data not shown).

The most prominent effect induced by the addition of transducin in the rhodopsin sample was the accumulation of metarhodopsin II. Namely, the difference spectrum obtained by irradiation of rhodopsin in the presence of transducin at −25 °C (curve 12′ in Figure 1e) displayed absorbance at 380 nm significantly larger than that obtained in the absence of transducin (curve 12′ in Figure 1d). Thus, a large amount of metarhodopsin II was accumulated in the presence of transducin. On the other hand, the presence of GTPγS caused little enhancement of the formation of metarhodopsin II even in the presence of transducin (curve 12′ in Figure 1f), suggesting that GTPγS can abolish the interaction between metarhodopsin II and transducin. These results are consistent with those reported previously (Hofmann, 1985), and also indicate that the coupling of rhodopsin intermediate(s) with transducin is monitored under our experimental conditions.

An interesting observation was that at each temperature (−35 or −25 °C), the spectral changes due to the thermal reactions of intermediates did not form an isosbestic point. Furthermore, the positive and negative maxima of the difference spectrum shifted to the blue as the time of incubation increased. These facts indicated that at least two conversion processes took place during the incubation at each temperature. The difference spectrum obtained by the long incubation at −35 °C had a positive maximum at about 440 nm (curve 10′ in Figure 1a), which was different from that (380 nm) obtained at −25 °C (curve 12′ in Figure 1d). Therefore, it is unlikely that the intermediate formed at the later stage of incubation at −35 °C is metarhodopsin II. The observed spectral changes do not fit the conventional scheme composed of canonical intermediates such as lumi, meta I, and meta II.

In order to estimate how many intermediates were present in the conversion process from lumi to meta II, we have analyzed the spectral changes by means of singular value decomposition followed by calculation of b-spectra and their apparent time constants (Figure 2).

The spectral changes observed at −35 °C were expressed by two b-spectra, indicating that the changes are not due to the single decay of lumi to meta I as previously inferred from the conventional warming−cooling experiments (Yoshizawa & Shichida, 1982). Since both b-spectra show no absorbance peaks below 400 nm (Figure 2a,b,c, upper and lower curves), these spectra do not reflect the formation of meta II. Thus, it is reasonable to speculate that at least two intermediates are present in the conversion process from lumi to meta II and they have absorption maxima located in between those of lumi and meta II. Hereafter, the two intermediates are referred to as meta I_a and meta I_b. The first b-spectrum reflects the decay of lumi to meta I_a and the second b-spectrum the decay of meta I_a to meta I_b.[2] It should be noted that the apparent time constant for the first b-spectrum was significantly smaller (18 times) than that for

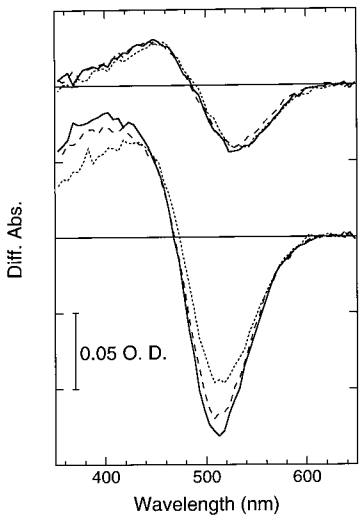

FIGURE 3: B-spectra obtained from the spectral changes observed at −35 °C. Upper and lower curves represent the first and second b-spectra. B-spectra of the samples containing only rhodopsin (sample A), rhodopsin + transducin (sample B), and rhodopsin + transducin + GTPγS (sample C) are represented by dotted, solid, and dashed curves, respectively. The b-spectra are normalized so that they represent the changes induced by the photoreaction of 1.0 absorbance of rhodopsin.

the second b-spectrum, suggesting that the two conversion processes were well separated.

The spectral changes observed at −25 °C were also expressed by two b-spectra (Figure 2d,e,f) with significant difference in the apparent time constant. The first b-spectra at −25 °C (Figure 2d,e,f, upper curves) were similar in shape to the second b-spectra at −35 °C (Figure 2a,b,c, lower curves), suggesting that the state formed at the later stage at −35 °C is similar to that formed at the early stage at −25 °C. This fact also suggests that lumi and meta I_a formed an equilibrium state and they behaved like a single intermediate during the conversion at −25 °C. The second b-spectrum showed the formation of metarhodopsin II (Figure 2d,e,f, lower curves).

We next compared b-spectra of sample A (containing only rhodopsin) with those of sample B (rhodopsin + transducin) and sample C (rhodopsin + transducin + GTPγS) (Figures 3 and 4). The b-spectra reflecting the conversion from lumi to meta I_a were similar among each other (Figure 3, upper curves), indicating that transducin as well as GTPγS does not affect the conversion process from lumi to meta I_a. On the other hand, as already inferred from the raw data of spectral changes (Figure 1d,e,f), the b-spectra reflecting the formation of meta II were greatly affected by transducin (Figure 4b). Transducin enhanced the accumulation of meta II, and GTPγS abolished the enhancement. Thus, it is likely that transducin can form a complex with meta II and dissociates from meta II through the GDP−GTP exchange reaction.

The shape of the b-spectrum reflecting the conversion from meta I_a to meta I_b (Figure 3, lower curves, and Figure 4a) also changed when transducin was present. Since transducin does not interact with meta I_a, meta I_b is the intermediate that interacts with transducin. The interesting observation is that the b-spectrum obtained in the presence of transducin and GTPγS (lower dashed curve in Figure 3 and dashed curve in Figure 4a) was similar in shape to that obtained in the presence of transducin (lower solid curve in Figure 3 and solid curve in Figure 4a), but different from that obtained in the absence of transducin (lower dotted curve in Figure 3 and dotted curve in Figure 4a). These results indicated that the addition of GTPγS had no effect on transducin binding to meta I_b. Therefore, we concluded that meta I_b interacts with transducin but does not induce the GDP−GTP exchange reaction in transducin.

To examine whether or not coexistence of Tα and T$\beta\gamma$ subunits is essential for the observed effects, we investigated the thermal reactions of rhodopsin intermediates in the presence of Tα or T$\beta\gamma$ subunit. The results showed that b-spectra were similar in shape to those obtained in the sample containing only rhodopsin (Figure 5). Thus, only the trimeric form of transducin can interact with meta I_b and meta II.

In order to estimate the absorption spectrum of meta I_b, we performed the following calculations using the first and second b-spectra at −25 °C (referred to as BS1 and BS2). The conversion process at −25 °C was expressed by two b-spectra, while four intermediates (lumi, meta I_a, meta I_b, and meta II) appeared in the process. These facts indicate the presence of a quasi-equilibrium state between lumi and meta I_a. Thus, the b-spectra are expressed by a linear combination of the spectra of these intermediates as follows:

$$\mathrm{BS1} = a_1(\alpha\epsilon_{\mathrm{lumi}} + \beta\epsilon_{\mathrm{meta\ I_a}}) + b_1\epsilon_{\mathrm{meta\ I_b}} + c_1\epsilon_{\mathrm{meta\ II}}$$

$$\mathrm{BS2} = a_2(\alpha\epsilon_{\mathrm{lumi}} + \beta\epsilon_{\mathrm{meta\ I_a}}) + b_2\epsilon_{\mathrm{meta\ I_b}} + c_2\epsilon_{\mathrm{meta\ II}}$$

where ϵ_{lumi}, $\epsilon_{\mathrm{meta\ I_a}}$, $\epsilon_{\mathrm{meta\ I_b}}$, and $\epsilon_{\mathrm{meta\ II}}$ are the absorption spectra of lumi, meta I_a, meta I_b, and meta II intermediates, respectively, and a_i, b_i, and c_i ($a_i + b_i + c_i = 0$, $i = 1, 2$) are the mole fractions of the respective intermediates. α and β are the constants derived from the equilibrium constant between lumi and meta I_a. The BS1 exhibited a positive maximum at the shorter wavelength (Figure 4a). This fact indicates that the absorption spectrum of meta I_b is blue-shifted from those of lumi and meta I_a, because BS1 reflects mainly the conversion from a mixture of lumi and meta I_a to meta I_b. Therefore, both the BS1 and BS2 at longer wavelengths originate from only lumi and meta I_a. Then we calculated the ratio of a_1 to a_2 from absorbances of the b-spectra in the wavelength region from 555 to 580 nm, where the ratios of absorbance were constant, while at shorter wavelengths they increased. The difference spectrum between meta I_b and meta II was calculated by subtracting the BS2 from the BS1 after normalizing the BS2 with the ratio. The calculated spectrum is shown in the inset of Figure 4b. Since meta II has no absorbance at the wavelength region longer than 420 nm, the absorption maximum of meta I_b is estimated to be 460 nm.

DISCUSSION

In the present study, we have identified two intermediates (meta I_a and meta I_b) in the conversion process of lumi to meta II and showed that the latter intermediate, meta I_b, interacts with transducin. This is the first experimental evidence that the intermediate formed before meta II has a

[2] Strictly, the first and the second b-spectra reflect the decay of lumi to a quasi-equilibrium state of lumi and its following intermediate (meta I_a) and that of the quasi-equilibrium state to an equilibrium state of lumi, meta I_a, and meta I_b, respectively.

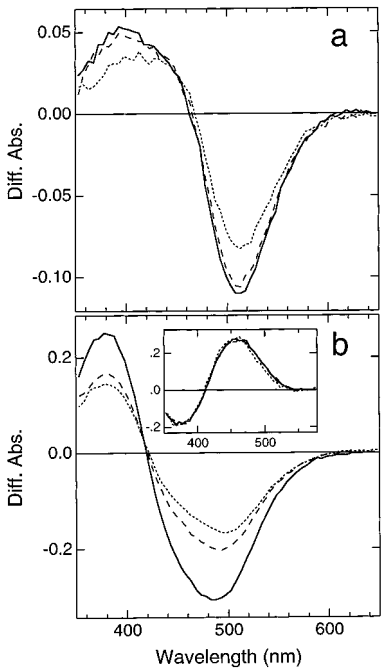

FIGURE 4: B-spectra obtained from the spectral changes observed at −25 °C. B-spectra of samples containing only rhodopsin (sample A), rhodopsin + transducin (sample B), and rhodopsin + transducin + GTPγS (sample C) are represented by dotted, solid, and dashed curves, respectively. (a) The first b-spectra. (b) The second b-spectra. Inset: Normalized difference spectra between meta I_b and meta II calculated from the b-spectra obtained from sample A (dotted curve), sample B (solid curve), and sample C (dashed curve). The b-spectra are normalized so that they represent the changes induced by the photoreaction of 1.0 absorbance of rhodopsin.

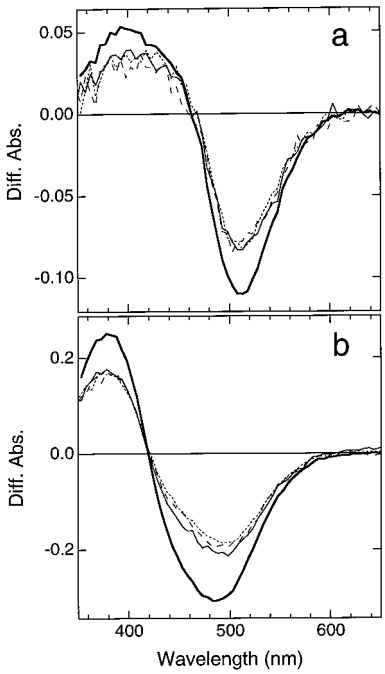

FIGURE 5: Effect of Tα or T$\beta\gamma$ on the thermal reactions of rhodopsin intermediates. B-spectra were calculated from the spectral changes observed at −25 °C using samples containing only rhodopsin (3.01 μM) (sample A), rhodopsin (3.01 μM) + Tα (9.60 μM) + T$\beta\gamma$ (9.60 μM) (sample B), rhodopsin (3.01 μM) + Tα (9.60 μM) (sample A+Tα), and rhodopsin (3.01 μM) + T$\beta\gamma$ (9.60 μM) (sample A+T$\beta\gamma$). B-spectra of sample A, sample B, sample A+Tα, and sample A+T$\beta\gamma$ are represented by dotted, thick, dashed, and thin curves, respectively. (a) The first b-spectra. Time constants of sample A, sample B, sample A+Tα, and sample A+T$\beta\gamma$ are 18.3, 23.7, 17.5, and 18.7 min, respectively. (b) The second b-spectra. Time constants of sample A, sample B, sample A+Tα, and sample A+T$\beta\gamma$ are 186.7, 295.0, 175.9, and 204 min, respectively. The b-spectra are normalized so that they represent the changes induced by the photoreaction of 1.0 absorbance of rhodopsin.

direct role to transfer a photon signal to transducin. On the other hand, the manner of interaction of meta I_b with transducin is different from that of meta II. The meta I_b binds to transducin but induces no GDP−GTP exchange reaction in transducin, while meta II can induce the exchange reaction. Therefore, the process in activating transducin by rhodopsin intermediates is composed of at least two steps. Since the transducin α or $\beta\gamma$ subunit alone showed no effect on the thermal reactions of rhodopsin intermediates, it is reasonable to speculate that only the heterotrimeric form of transducin can interact with rhodopsin intermediates (Figure 6).

Molecular Mechanism Leading to Activation of Transducin. The absorption maximum of meta I_b is 460 nm, which is similar to that of meta I_a (480 nm; Imai et al., 1994) but considerably red-shifted from that of meta II (380 nm). These results suggest that meta I_b has a protonated retinylidene Schiff base as its chromophore, like meta I_a. Thus, it is unlikely that deprotonation of the chromophore is a prerequisite for the protein conformation responsible for transducin binding, although the further change in protein conformation including Schiff base deprotonation is necessary for the GDP−GTP exchange reaction in transducin.

Conformational changes of the protein other than the Schiff base region have been frequently demonstrated (Shichida, 1986; Shichida et al., 1987, 1991; Albeck et al., 1989; Ganter et al., 1989; Einterz et al., 1990; Okada et al., 1991). Although the chromophore is photoisomerized by movement of half of the polyene chain containing the Schiff base (Shichida et al., 1987), subsequent changes occur in the protein near the cyclohexenyl ring of the chromophore (Okada et al., 1991). Then the interaction present in the original rhodopsin state between the 9-methyl group of the chromophore and the surrounding protein disappears (Shichida et al., 1991). The removal of the β-ionone ring or the 9-methyl group of the chromophore resulted in reduced activation of transducin (Ganter et al., 1989; Jäger et al., 1994; Corson et al., 1994; Morrison et al., 1995), indicating that the changes in protein conformation near the β-ionone ring and the 9-methyl group are necessary for the formation of the active state of rhodopsin. Recent cross-linking and mutagenesis studies indicated that the β-ionone ring is situated near Trp-265 and Leu-266 in helix 6 (Zhang et al., 1994) and the 9-methyl group is surrounded by the residues including Gly-121 in helix 3 and Phe-261 in helix 6 (Han et al., 1996; Shieh et al., 1997). Furthermore, a change in the orientations of helices 3 and 6 has been implicated as a key element for the coupling between the active states of rhodopsin and transducin (Sheikh et al., 1996; Farrens et al., 1996; Acharya et al., 1997). Thus, the activation mechanism of transducin by rhodopsin is speculated as follows: Photoisomerization of the rhodopsin chromophore causes conformational changes of the amino acid residues situated near the β-ionone ring and 9-methyl group of the chromophore, resulting in tilt of helices of 3 and 6 to give rise to a specific conformation of the cytoplasmic loop to interact with transducin. Then the changes in conformation of the rhodopsin−transducin complex including the Schiff base

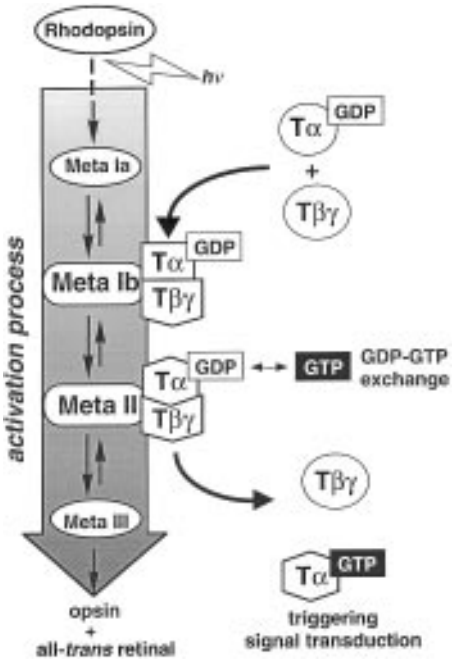

FIGURE 6: Schematic drawing of the process in activating transducin by rhodopsin intermediates. On absorption of light, rhodopsin converts to meta I_b through several intermediates. Meta I_b binds to transducin but induces no GDP−GTP exchange reaction in transducin. The GDP−GTP exchange reaction occurs at the stage of meta II.

region of rhodopsin could occur to accomplish a GDP−GTP exchange reaction.

Reaction Kinetics of Intermediates. From the differences in b-spectra, we concluded that meta I_b and meta II have a direct role in activating transducin. If these intermediates can interact with transducin, the rate constants of the conversions could also be affected by transducin. The process observed in the presence of both transducin and GTPγS should be complicated, because it contains several binding and dissociation reactions in addition to the conversions among intermediates. Thus, we mainly discuss the difference in reaction kinetics at −25 °C between the sample containing only rhodopsin and that containing rhodopsin and transducin, according to the reaction scheme shown as follows:

$$(\text{lumi} \underset{\text{fast}}{\overset{\text{fast}}{\rightleftharpoons}} \text{meta } I_a) \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{meta } I_b \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \text{meta II}$$

where k_{ij} denotes the rate constant for the transition from intermediate i to intermediate j. If one can assume that the interactions between intermediates and transducin occur with relatively large rate constants, the apparent rate constants (λ_1 and λ_2) obtained by the experiments are related to the rate constants by the equations:

$$\lambda_1 = (p + q)/2$$

$$\lambda_2 = (p - q)/2$$

where $p = (k_{12} + k_{21} + k_{23} + k_{32})$ and $q = [p^{2-}4(k_{12}k_{23} + k_{21}k_{32} + k_{12}k_{32})]^{1/2}$. Our experimental results showed that the apparent rate constants for the first b-spectra (λ_1's) are not so different between these samples, while those for the second b-spectra (λ_2's) are significantly different. Since the experiments indicated that each λ_1 is significantly larger than the respective λ_2, λ_1 and λ_2 are approximated as sums of the rate constants for the forward and back-reactions, that is, ($k_{12} + k_{21}$) and ($k_{23} + k_{32}$), respectively. Thus, the small difference in λ_1 between these samples suggests that the reaction affected by transducin (probably the back-reaction from meta I_b to meta I_a) has a rate constant relatively smaller than that unaffected by the transducin (the forward reaction from meta I_a to meta I_b). That is, the equilibrium constant ($K_1 = k_{12}/k_{21}$) could be large even in the absence of transducin. On the other hand, k_{23} and k_{32} could change by the addition of transducin, resulting in a significant change of the λ_2 in these samples.

When spectra of all the intermediates are known, the rate constants are calculated from b-spectra and the concentrations of intermediates at adequate time (Thorgeirsson et al., 1993). However, only the spectra of lumi and meta I_a were available from the low-temperature spectroscopy at −80 and −60 °C (Imai et al., 1994). The spectrum of meta II calculated in our previous paper (Imai et al., 1994) might be a mixture of meta I_b and meta II. Thus, the rate constants were preliminary estimated by a fitting procedure. In the fitting procedure, two equilibrium constants ($K_1 = k_{12}/k_{21}$, $K_2 = k_{23}/k_{32}$) and initial concentrations of intermediates were set as parameters. Then the spectrum of the first intermediate (a mixture of lumi and meta I_a) calculated using these parameters was best fitted with the spectrum of a mixture of lumi and meta I_a. The estimated values of k_{12}, k_{21}, k_{23}, and k_{32} were 6.3, 2.1, 0.72, and 0.38 ($\times 10^{-4}$ s^{-1}) in the sample containing rhodopsin, and 6.6, 0.34, 0.54, and 0.051 ($\times 10^{-4}$ s^{-1}) in the sample containing both rhodopsin and transducin, respectively. These results indicated that the reaction from meta I_a to meta I_b (k_{12}) was not affected by transducin, while the rate constant for the back-reaction from meta I_b to meta I_a (k_{21}) was 6 times smaller when transducin was present. Furthermore, the rate constant for the forward reaction of meta I_b to meta II (k_{23}) became slightly smaller in the presence of transducin, while that of the back-reaction (k_{32}) became about 7 times smaller. Similar analysis was adopted to the experimental results obtained from the sample containing rhodopsin, transducin, and GTPγS, and showed that the rate constant for the back-reaction from meta I_b to meta I_a (0.37) was similar to that obtained in the presence of transducin. The back-reaction from meta II to meta I_b (rate constant = 0.18) was significantly accelerated in the presence of GTPγS. All these results strongly suggested that transducin stabilizes meta I_b and meta II, and that GTPγS abolishes the interaction between meta II and transducin.

Since our experiments were performed at relatively low temperature (−25 or −35 °C), it is important to examine whether or not similar reactions occur at a physiological temperature. The presence of multiple forms of meta I (Thorgeirsson et al., 1992, 1993) and meta II (Straume et al., 1990; Arnis & Hofmann, 1993) has been frequently reported. Therefore, some form(s) of these intermediates might correspond to meta I_b detected by low temperature. Our future research will investigate the temperature dependency of the formation of meta I_b and its interaction with transducin.

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