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Two-Step Mechanism of Interaction of Rhodopsin Intermediates with the C-Terminal Region of the Transducin α-Subunit

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Rhodopsin is a prototypical G-protein–coupled receptor that contains 11-*cis*-retinal as a light-absorbing chromophore. Light causes conformational changes in the protein moiety through *cis-trans* isomerization of the chromophore, which leads to the formation of G-protein–interacting states. Our previous studies indicated that there are two intermediate states of rhodopsin, Meta Ib and Meta II, which interact differently with retinal G-protein transducin (Gt) [S. Tachibanaki, H. Imai, T. Mizukami, T. Okada, Y. Imamoto, T. Matsuda, Y. Fukada, A. Terakita, and Y. Shichida (1997) *Biochemistry* 36, 14173–14180]. Here we demonstrate that the interactions of Gt with these intermediates in the absence of GTPγS can be mimicked by the C-terminus 11-amino acid peptide (340–350) of the α-subunit of Gt (Gtα), suggesting that the C-terminal region of Gtα plays important roles in the interaction with rhodopsin intermediates. Replacement of either of the two leucine residues (Leu344 and Leu349) in the peptide with alanine caused the loss of the interaction with Meta II. However, the interaction with Meta Ib was abolished only when both residues were replaced. These results indicate that rearrangement of the C-terminal region of Gtα after the binding of a rhodopsin intermediate is necessary for the GDP-GTP exchange reaction on Gtα.

Key words: G protein, G-protein–coupled receptor, intermediate, protein–protein interaction, rhodopsin.

Abbreviations: Gt, retinal G-protein transducin; Gtα, α-subunit of transducin; GPCR, G-protein coupled receptor; ROS, rod outer segment; CHAPS, 3-[(3-cholamidopropyl)dimethyl- ammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; HEPES, *N*-(2-hydroxy- ethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethansulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); SVD, singular value decomposition; FTIR, Fourier transform infrared.

The visual transduction process in rod photoreceptor cells begins with light absorption by rhodopsin, which is a member of the G-protein-coupled receptor (GPCR) family, and contains 11-*cis*-retinal as a light-absorbing chromophore (*1*, *2*). Through a protonated Schiff base linkage, 11-*cis*-retinal is bound to the lysine residue at position 296 in the seventh helix. Light causes conformational changes in the protein moiety of rhodopsin through *cis-trans* isomerization of the chromophore, which leads to activation of a G-protein–mediated signal transduction cascade that eventually generates an electrical response of the photoreceptor cells. Because rhodopsin is a unique GPCR that can be activated by light, a physical stimulus, the molecular mechanism of G-protein activation by rhodopsin was thought to be somewhat different from those in the case of other ligand-binding GPCRs. However, accumulated evidence has revealed that the chromophore of rhodopsin acts as an inverse agonist (*3*, *4*), and light plays a role by converting the inverse agonist into the agonist all-*trans*-retinal within the protein moiety, suggesting that the G-protein activation mechanism is similar for rhodopsin and other GPCRs. Therefore, elucidation of the mechanism of activation of transducin by rhodopsin would provide an insight into the common mechanism of G-protein activation by GPCRs.

The conformational changes in the protein moiety of rhodopsin after absorption of light can be monitored by means of spectroscopic techniques, because the chromophore of rhodopsin acts as an intrinsic spectroscopic probe in the rhodopsin molecule. So far, at least seven intermediate states have been identified in the photobleaching process of rhodopsin [Fig. 1A, (*2*)]. Among the intermediate states, the state named metarhodopsin II (Meta II), which forms as an equilibrium mixture with metarhodopsin I (Meta I) (*5*), was thought to be a crucial state that binds to and activates retinal G-protein transducin (Gt). That is, the addition of Gt to the equilibrium mixture of Meta II (~380 nm) and Meta I (~480 nm) caused a shift of the equilibrium in the favor of Meta II, resulting in the formation of a large amount of Meta II (the increased fraction is referred to as "extra Meta II") (*6*, *7*). The extra formation of Meta II was abolished upon the addition of GTP or its analogue GTPγS to the pigment-transducin mixture (*7*, *8*). These results were interpreted as meaning that Gt can form a complex with Meta II in the absence of GTP, resulting in a shift of the equilibrium, and it dissociates from Meta II through the

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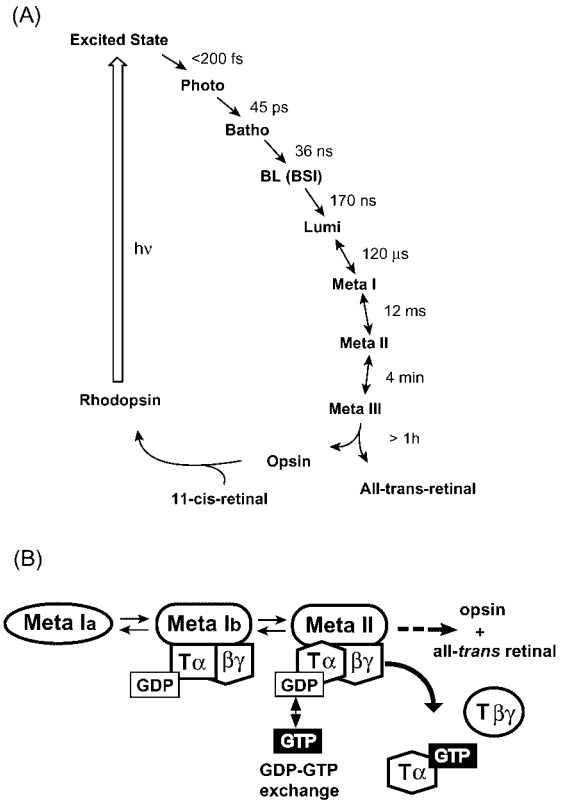

Fig. 1. **(A) Photobleaching of bovine rhodopsin.** The time constants of the transitions between intermediates observed at room temperature are shown on the right of the arrows (*2*). (B) Detailed spectroscopic analysis of the intermediate states that interact with Gt at –25°C. On absorption of light, rhodopsin is converted to Meta I through the several intermediates shown in (A). We found two states of Meta I (Meta Ia and Ib) on detailed kinetic analysis. Meta Ia is converted to Meta Ib and then to Meta II. Finally, an equilibrium state composed of these three states is formed at –25°C. Low-temperature spectroscopic analysis showed that Meta Ib binds to Gt but induces no GDP-GTP exchange reaction on Gt, while the GDP-GTP exchange reaction occurs at the stage of Meta II (*9*).

GDP-GTP exchange (activation) reaction in the presence of GTP. However, the experimental results previously described do not rule out the possibility that another intermediate(s) binds Gt with an affinity lower than that of Meta II. In fact, our recent low-temperature spectroscopic investigations showed that there are two states of metarhodopsin I, Meta Ia and Ib, and that the conversion of Meta Ib to Meta II is decelerated in the presence of Gt, suggesting that Meta Ib is transiently trapped by Gt (Fig. 1B). The transient trapping of Meta Ib by Gt was also observed in the presence of GTPγS (*9*), indicating that Meta Ib can bind to Gt but induces no GDP-GTP exchange (activation) reaction on the α-subunit of Gt (Gtα). Therefore, elucidation of the molecular mechanism that causes the difference in the interaction mode between Meta Ib and Meta II would be important for furthering our understanding of the mechanism of activation of Gt by rhodopsin.

In the present study, we attempted to elucidate the difference in interaction mode between Meta Ib and Meta II. Because the peptide comprising the C-terminal 11 amino acids of Gtα plays a similar role to the whole G protein in the interaction with Meta II (*10*), it was of interest to examine whether or not the peptide plays a similar role in the interaction with Meta Ib. The current findings clearly show that the spectral changes observed in the presence of the peptide are almost the same as those observed in the presence of the whole G protein. These results clearly show that the C-terminal region of Gtα is sufficient to mimic the spectral changes of the rhodopsin intermediates induced by the whole G protein. Furthermore, replacement of either of the two leucine residues (Leu344 and Leu349) in the peptide with alanine caused the loss of the interaction with Meta II but the ability to interact with Meta Ib was retained. Replacement of both leucine residues caused the loss of the interaction with Meta Ib. On the basis of these experimental results, the mode of interaction of the C-terminus region of Gtα with rhodopsin intermediates is discussed.

EXPERIMENTAL PROCEDURES

Preparation of Bovine Rhodopsin and Gt—Rhodopsin was extracted from the rod outer segments (ROS) of bovine retinas, with CHAPS as a detergent, and was purified by column chromatography (*11*). Briefly, rhodopsin extracted with buffer A [50 mM HEPES, 1% (w/v) CHAPS, 140 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF, 1 μg/ml leupeptin, 12.5 KIU/ml aprotinin, 1 mM DTT, pH 7.5, at 4°C] was applied to a ConA-Sepharose (Amersham Pharmacia) column, from which rhodopsin was eluted with buffer B [50 mM HEPES, 0.6% (w/v) CHAPS, 0.8 mg/ml PC, 140 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF, 1 μg/ml leupeptin, 12.5 KIU/ml aprotinin, 1 mM DTT, pH 7.5, at 4°C] supplemented with 0.2 M methyl α-D-mannopyranoside. The concentration of rhodopsin in the sample was estimated from its absorbance at the maximum (Molar extinction coefficient = 41,200) (*12*, *13*). All procedures were performed at 4°C and under dim red light.

Gt was purified from fresh bovine retinas according to the method reported by Fukada *et al*. (*12*). Briefly, Gt was extracted from the bovine ROS membranes with hypotonic buffer C (5 mM Tris, 0.5 mM $MgCl_2$, 0.1 mM PMSF, 1 μg/ml leupeptin, 12.5 KIU/ml aprotinin, 1 mM DTT, pH 7.2, at 4°C) supplemented with 100 μM GTP. The sample was applied to a Blue-Sepharose (Amersham Pharmacia) column that had been equilibrated with buffer B. Gtα bound to the column, while the βγ subunit (Gtβγ) passed through the column. Thus, Gtα was eluted from the column with buffer D (10 mM MOPS, 600 mM NaCl, 2 mM $MgCl_2$, 1 μg/ml leupeptin, 12.5 KIU/ml aprotinin, 1 mM DTT, pH 7.5, at 4°C). The flow-through fractions containing Gtβγ were applied to a DEAE-Toyopearl 650S (Tosoh) column, from which Gtβγ was eluted with buffer C. Equal amounts of Gtα and Gtβγ were then mixed, and the buffer was exchanged with 50 mM HEPES, 140 mM NaCl, 2 mM $MgCl_2$, 1 μg/ml leupeptin, 12.5 KIU/ml aprotinin, 1 mM DTT (pH 7.5 at 4°C) using a gel filtration column (PD-10, Pharmacia Biotech), followed by concentration to about 150 μM with an ultrafiltration membrane (Centricon-30, Amicon). The Gtα prepared under our experimental conditions is in a GDP-bound form (*14–17*) and can form a heterotrimer with Gtβγ (*12*). Protein concentra-

tions were estimated by the method of Bradford (*18*) with bovine serum albumin as a standard. The samples were stored at –80°C until use.

Preparation of Synthetic Peptides—An 11-mer synthetic peptide at the C-terminus of Gtα (340–350; IKENLKDCGLF) and three derivatives of it with substitution of Leu344 and/or Leu349 with alanine (IKENAKDCGLF, IKENLKDCGAF, and IKENAKDCGAF) were produced by Chiron Technologies, and purchased from Kurabo Industries. The final purification of the synthesized peptides by reverse-phase HPLC was carried out in a water-acetonitrile gradient system containing 0.1% trifluoroacetic acid. The purity of the peptide samples examined by reverse-phase HPLC was higher than 95%. Each weighed, lyophilized peptide was dissolved in Gt preparation buffer (50 mM HEPES, 140 mM NaCl, 2 mM $MgCl_2$, 1 μg/ml leupeptin, 12.5 KIU/ml aprotinin, 1 mM DTT, pH 7.5, at 4°C) to obtain a final concentration of 20 mM.

Time-Resolved Spectroscopy—Absorption spectra were recorded with a photodiode-array spectrophotometer that was specially constructed based on a commercial spectrophotometer, Shimadzu MultiSpec-1500 PDA. This spectrophotometer can continuously record a spectrum (750 to 330 nm) with a time resolution of 1 s after irradiation of rhodopsin. To record spectra at 0°C, an optical cryostat (Oxford, Optistat) with an optical cell of 1-cm light path was placed in the sample compartment of the spectrophotometer. The sample temperature was regulated to within 0.1°C with a temperature controller (Oxford, ITC502) attached to the cryostat. Each sample was irradiated with light from a 1-kW tungsten-halogen lamp (Rikagaku-Seiki). The wavelength of the irradiated light was controlled with a glass cutoff filter (Toshiba). The thermal reactions of the intermediates initiated by irradiation of the rhodopsin sample were monitored by recording the absorption spectra at intervals of 1–30 s until the reactions were almost saturated. The amount of rhodopsin photoconverted to the intermediates by the irradiation was estimated according to the method reported by Tachibanaki *et al*. (*9*).

RESULTS

Spectroscopic Observations of the Interactions of Meta Ib and II with Gt at 0°C—On low-temperature time-resolved spectroscopy, we previously identified a new intermediate state, Meta Ib, that interacts with Gt in a manner different from Meta II (*9*, *19*). Because we observed the interactions of Meta Ib and Meta II with Gt in the temperature range of –20 to –35°C in the previous study, we tried to obtain evidence that the interactions really occur at a higher temperature. Therefore, we newly developed a photodiode-array (PDA) spectrophotometer that can continuously record a spectrum (from 750 nm to 330 nm) with a time resolution of 1 s, *i.e.* about 200 times higher than that of the conventional spectrophotometer used for the previous study. Figure 2 shows data obtained in typical experiments with a series of samples containing 27% glycerol. Curves 1 in Fig. 2, A, B, and C, shows the absorption spectra of three samples containing only rhodopsin (5 μM) (Fig. 2A), rhodopsin (5 μM) and Gt (20 μM) (Fig. 2B), and rhodopsin (5 μM), Gt (20 μM), and GTPγS (400 μM) (Fig. 2C), respectively. These samples exhibit absorption maxima at about 500 nm, which are similar to those reported in the literature. Upon irradiation with orange light at 0°C, the spectral changes due to the conversion of Meta I to Meta II were observed (curves 2 to 11). Namely, after the blue-shift of the absorption spectrum (curve 2) due to the formation of a mixture of the intermediates, an increase in absorbance at about 380 nm with a concurrent decrease in absorbance at about 480 nm was observed. Fifteen minutes after irradiation (curve 11), the spectral changes were

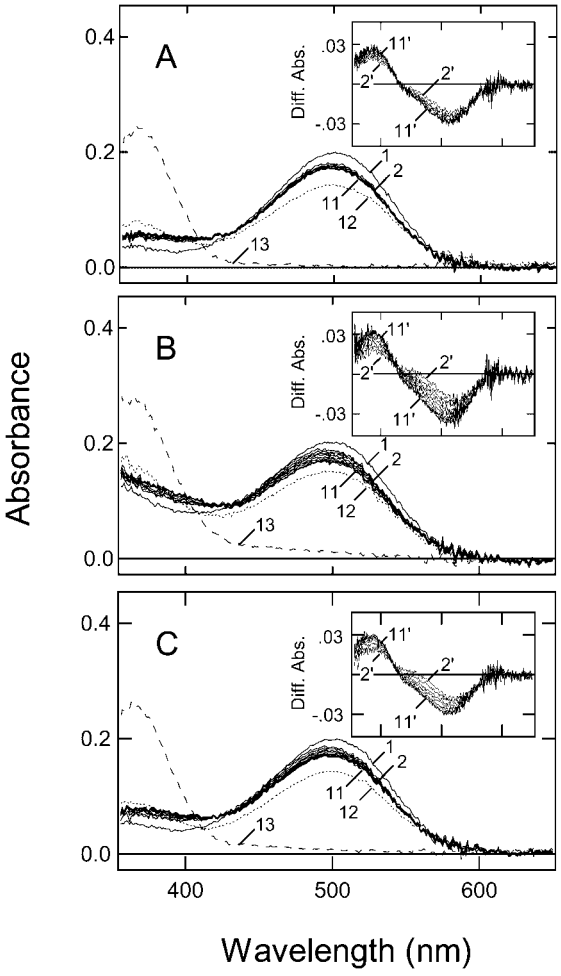

Fig. 2. **Effect of Gt on the thermal reaction from Meta Ib to Meta II.** Three samples, which contained (A) rhodopsin (5 μM), (B) rhodopsin (5 μM) and Gt (20 μM), and (C) rhodopsin (5 μM), Gt (20 μM), and GTPγS (400 μM), respectively, were mixed with glycerol to obtain a final concentration of 27% (v/v) (curves 1) and then irradiated with > 500-nm light for 1 s (curves 2) at 0°C, followed by incubation at this temperature. Absorption spectra recorded at 2, 3, 4, 8, 16, 40, 100, 300, and 900 s after the irradiation are shown (curves 3–11, respectively). The sample was then warmed to 10°C, followed by the addition of a 1/20 vol. of a neutralized 1 M hydroxylamine solution to decompose the intermediates present in the sample into retinal oxime and opsin. The sample was then cooled again to 0°C (curves 12) and irradiated with >500-nm light for 20 min (curves 13) for conversion of the residual rhodopsin in the sample to retinal oxime and opsin. Insets: Difference spectra (curves 2′–11′) calculated by subtracting the spectra recorded before irradiation (curves 1) from those recorded after incubation at adequate times after irradiation (curves 2–11).

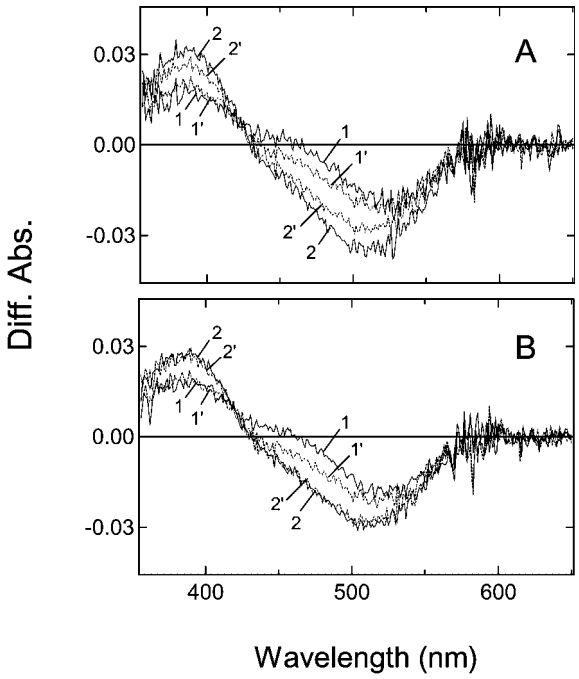

Fig. 3. **Effect of Gt on the thermal reaction from Meta Ib to Meta II.** Difference spectra calculated by subtracting the spectra recorded before irradiation from those recorded immediately after irradiation (curves 1 or 1′), and that recorded after 15-min incubation (curves 2 or 2′). These spectra are normalized as to the amount of photoactivated rhodopsin. Curves 1 and 2 were obtained for a sample containing 5 μM rhodopsin + 20 μM Gt (A) or 5 μM rhodopsin + 20 μM Gt + 400 μM GTPγS (B). Curves 1′ and 2′ were for a sample containing only 5 μM rhodopsin.

apparently finished, suggesting the formation of an equilibrium state between Meta I and Meta II. Because the spectra contain the contribution of unphotolysed rhodopsin (curve 12), the spectral changes are enlarged in the insets, where the changes are expressed as difference spectra (curves 2′–11′) calculated by subtracting the spectra recorded before the irradiation (curve 1) from those recorded at selected times afterwards (curves 2–11). Singular value decomposition (SVD) analysis of the spectral changes indicated that these changes can be expressed by a single exponential process including the decay of the intermediate with a difference absorption maximum at 480 nm and the formation of the intermediate with a difference absorption maximum at 380 nm with time constants of 2.7 s (sample A), 9.1 s (sample B), and 4.0 s (sample C). Based on these spectral and kinetic properties together with the previously published results obtained at –20°C (*19*), we assigned the process as the last reaction before the formation of the equilibrium between Meta I and Meta II, namely, the transition from Meta Ib to Meta II.

We observed that the presence of Gt caused the transient trapping of Meta Ib and the formation of extra Meta II (Fig. 2B). That is, the spectrum obtained at 1 s after the irradiation (curve 1 in Fig. 3A, same as curve 2′ in Fig. 2B) exhibited greater absorbance around 480 nm compared to the spectrum obtained in the absence of Gt (curve 1′ in Fig. 3A, same as curve 2′ in Fig. 2A), indicating the slowed decay of Meta Ib in the presence of Gt. The spectrum recorded 15 min after the irradiation (curve 2 in Fig. 3A, same as curve 11′ in Fig. 2B) exhibited greater absorbance at 380 nm compared to the spectrum obtained in the absence of Gt (curve 2′ in Fig. 3A, same as curve 11′ in Fig. 2A), indicating an excess amount of Meta II was accumulated in the presence of Gt. These results indicated that Gt interacts with Meta Ib and II, thereby changing the thermal behavior of the intermediates. The addition of GTPγS abolished the excess formation of Meta II but did not affect the transient trapping of Meta Ib by Gt. The spectrum obtained at 1 s after the irradiation (curve 1 in Fig. 3B, same as curve 2′ in Fig. 2C) was the same in shape as that obtained in the presence of Gt (curve 1 in Fig. 3A), while that recorded at 15 min after the irradiation (curve 2 in Fig. 3B, same as curve 11′ in Fig. 2C) was the same as that recorded in the absence of Gt (curve 2′ in Fig. 3B, same as curve 11′ in Fig. 2A).

To analyze the mode of interaction of Gt with the two intermediates kinetically, we plotted, for these samples, the changes in absorbance at 480 nm as a function of the incubation time after the irradiation (Fig. 4A). In comparison with the sample containing only rhodopsin (open circles in Fig. 4A), the time profile of the sample containing rhodopsin and Gt (solid circles in Fig. 4A) showed greater absorbance until 10 s incubation and lower absorbance after 10 s. The former change is due to the transient trapping of Meta Ib, and the latter to the formation of extra Meta II. The formation of extra Meta II was abolished by the addition of GTPγS, where only the greater absorbance up to 10-s incubation was observed (solid triangles in Fig. 4A). All of these results are similar to those obtained at –20 to –35°C on low-temperature time-resolved spectroscopy (*9*, *19*). Therefore, we conclude that the interaction of Gt with Meta Ib can be observed over a wide temperature range, at least under our experimental conditions.

During the experiments, we found that the interactions of Gt with Meta Ib and Meta II could be phenomenologically separated by changing the glycerol concentration in the sample. The addition of a large amount of glycerol to a sample shifts the equilibrium between Meta I and Meta II toward Meta II (*5*), thereby resulting in less prominent observation of the formation of extra Meta II (Fig. 4B). On the other hand, the effect of Gt on the formation of extra Meta II was observed more precisely for a sample containing a low concentration of glycerol, where the equilibrium favors Meta I, although the decay of Meta Ib was too rapid to be detected (Fig. 4C). Thus, to investigate the interactions of Gt with the two intermediates separately, we used these experimental conditions for the experiments described in the following sections.

Interaction of Meta Ib and Meta II with the C-Terminal Peptide of Gtα—It has been reported that the C-terminal 11 amino acid residues of Gtα form extra Meta II, as the whole Gt does (*10*). Thus, to determine whether or not the C-terminal 11 amino acid residues also transiently trap Meta Ib, we compared the thermal reactions of Meta Ib in the presence and absence of the C-terminal peptide (peptide LL, Fig. 5). Figure 6A and B show the spectral changes due to conversion of Meta Ib to Meta II in the absence and presence of the peptide, respectively, and the initial and final spectra of these changes are superim-

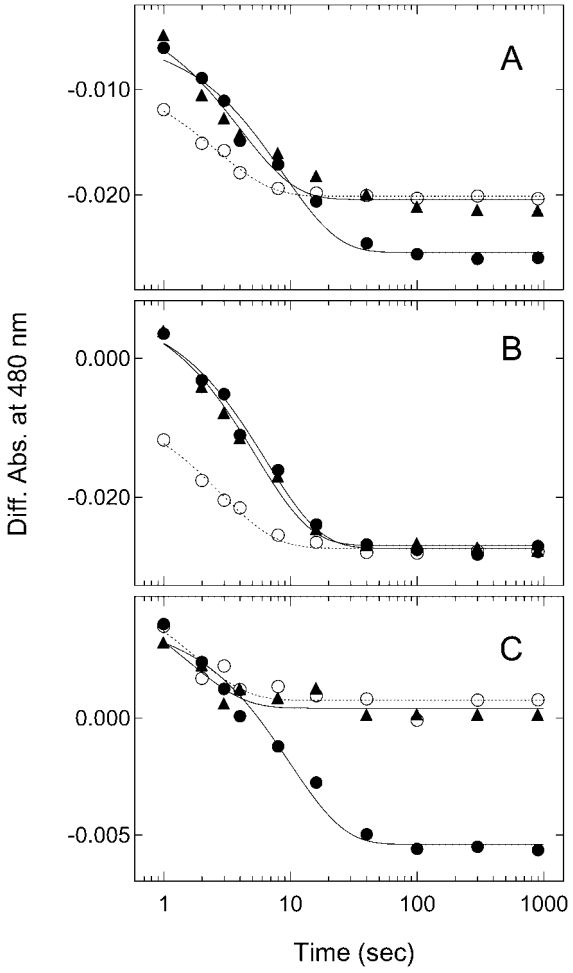

Fig. 4. **Effect of Gt on the time profile of the absorbance change at 480 nm.** (A) Time courses of absorbance changes at 480 nm during incubation of irradiated samples containing rhodopsin (open circles), rhodopsin + Gt (solid circles), and rhodopsin + Gt + GTPγS (solid triangles). The solid and dotted curves are the fitted single exponential curves with time constants of 2.7 s (open circles), 9.1 s (solid circles), and 4.0 s (solid triangles). (B) Time courses of absorbance changes at 480 nm obtained for samples containing rhodopsin (open circles), rhodopsin + Gt (solid circles), and rhodopsin + Gt + GTPγS (solid triangles). All samples were mixed with glycerol at a final concentration of 58% (v/v). The solid and dotted curves are the fitted single exponential curves with time constants of 3.4 s (open circles), 6.2 s (solid circles), and 5.3 s (solid triangles). (C) Time courses of absorbance changes at 480 nm obtained for samples containing rhodopsin (open circles), rhodopsin + Gt (solid circles), and rhodopsin + Gt + GTPγS (solid triangles). All samples were mixed with glycerol at a final concentration of 10% (v/v). The curves are the fitted single exponential curves with time constants of 1.7 s (open circles), 9.7 s (solid circles), and 1.6 s (solid triangles).

posed in Fig. 6C. The presence of the peptide (curve 1 in Fig. 6C, same as curve 1 in Fig. 6B) exhibited greater absorbance around 480 nm compared to the spectrum obtained in the absence of the peptide (curve 1′ in Fig. 6C, same as curve 1 in Fig. 6A), indicating that Meta Ib is transiently trapped in the presence of the peptide. The spectrum recorded 15 min after the irradiation (curve 2 in Fig. 6C, same as curve 10 in Fig. 6B) exhibited greater absorbance at 380 nm due to the formation of extra Meta II compared to the spectrum obtained in the absence of

	340										350	
Gt	:I	K	E	N	L	K	D	C	G	L	F	(LL)
Gi	:I	K	N	N	L	K	D	C	G	L	F	
Go	:I	A	N	N	L	R	G	C	G	L	F	
Gq	:L	Q	L	N	L	K	E	Y	N	L	V	
Gs	:Q	R	M	H	L	R	Q	Y	E	L	L	
AL	:I	K	E	N	A	K	D	C	G	L	F	(AL)
LA	:I	K	E	N	L	K	D	C	G	A	F	(LA)
AA	:I	K	E	N	A	K	D	C	G	A	F	(AA)

Fig. 5. **Gt-derived peptide and its analogues.** Upper panel: Amino acid sequences of the C-terminal regions of G protein α subunits. Conserved Leu-344 and Leu-349 are boxed. Lower panel: Sequences of the three alanine-replaced peptides used in the present study.

the peptide (curve 2′ in Fig. 6C, same as curve 10 in Fig. 6A). Figure 6D shows the time course of the absorbance change at 480 nm for the sample containing rhodopsin and the peptide (solid circles), and that containing only rhodopsin (open circles). Similar to the experiments involving the whole Gt (Fig. 3A), the experiments involving the C-terminal peptide clearly showed the transient trapping of Meta Ib and the formation of extra Meta II. Therefore, we conclude that the C-terminal region of Gtα plays an important role in the interaction with rhodopsin intermediates.

We next investigated the mechanism of the interaction between rhodopsin intermediates and the C-terminal region of Gtα. Because two leucine residues (L344 and L349 in Gtα) in the C-terminal region are highly conserved among all G protein subtypes (Fig. 5) and their mutation has been confirmed to prevent activation by rhodopsin (*20*), it is of interest to investigate the roles of these residues in the mechanisms of the interaction with rhodopsin intermediates. For this purpose, we prepared three analogue peptides in which each of the leucine residues at positions 344 and 349 or both residues were replaced with alanine (Fig. 5), and then subjected them to investigation of the thermal reactions.

As expected from the results of biochemical studies on G-protein activation by Meta II (*20*), replacement of either of the two leucine residues (Leu344 and Leu349) in the peptide with alanine caused the loss of the formation of extra Meta II (peptides AL and LA; Fig. 7A). On the other hand, replacement of Leu344 (AL) caused a slight decrease in the ability to transiently trap Meta Ib, whereas that of the Leu349 (LA) caused a 75% loss of the ability (Fig. 7B). Replacement of both the leucine residues (AA) caused a complete loss of the interaction even at the Meta Ib stage (Fig. 7, A and B). These results indicate that the interaction mode of the peptides differs between rhodopsin intermediates, suggesting that some conformational changes occur during the process of conversion from Meta Ib to Meta II. Figure 8 shows the relationship between the peptide concentration and transient trapping of Meta Ib. Peptide AL exhibited slightly weaker effect on the transient trapping (EC_{50} = 130 ± 14 μM) than peptide LL (EC_{50} = 90 ± 5 μM). Peptide LA (EC_{50} = 530 ± 25 μM) showed a significantly weaker effect

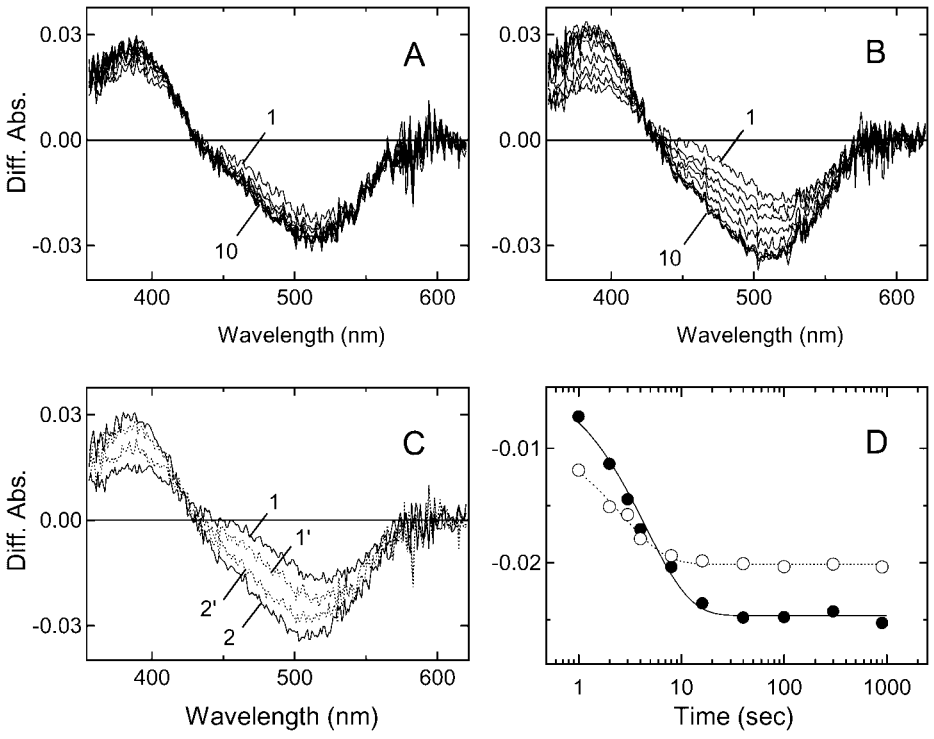

Fig. 6. **Interaction of the Gt-derived peptide with rhodopsin.** (A and B) Difference spectra calculated by subtracting the spectra recorded before irradiation from those recorded after irradiation, and incubation at 0°C for 1, 2, 3, 4, 8, 16, 40, 100, 300, and 900 s, respectively (curves 1–10). A rhodopsin (5 μM)-27% (v/v) glycerol mixture was irradiated with >500-nm light for 1 s at 0°C in the presence (A) or absence (B) of 500 μM peptide LL. (C) Difference spectra calculated by subtracting the spectra recorded before irradiation from those recorded immediately after irradiation (curve1 or 1′), and that recorded after 15-min incubation (curve 2 or 2′). Curves 1 and 2 were obtained for a sample containing 5 μM rhodopsin and 500 μM peptide LL. Curves 1′ and 2′ are for a sample containing only 5 μM rhodopsin. (D) Time courses of absorbance changes at 480 nm during the incubation of irradiated samples containing rhodopsin (open circles) and rhodopsin + peptide LL (solid circles). The curves are the fitted single exponential curves with time constants of 2.7 s (open circles) and 4.9 s (solid circles).

than peptides AL and LL, while peptide AA showed no effect under the experimental conditions used. In other words, with the single replacement of L344, the affinity for Meta Ib was retained but there was no affinity for Meta II. Replacement of L349 decreased the affinity for both Meta Ib and Meta II, suggesting the mechanism of the interaction of the C-terminal region of Gtα with Meta Ib differs from that with Meta II.

DISCUSSION

In the present study, we demonstrated the presence of the previously reported rhodopsin intermediate Meta Ib, which binds to Gt but induces no GDP-GTP exchange reaction on Gtα, in a temperature range higher than that previously reported. The decay time constant of Meta Ib at 0°C in the absence of glycerol was estimated to be about 500 ms by extrapolation of the time constants measured at eight different concentrations of glycerol to 0% glycerol. Three of the data sets are shown in Fig. 4.

This value is similar to the previously reported formation time constant of Meta II at 0°C in ROS (*21*, *22*). We also showed that the effect of Gt on the thermal behavior of Meta Ib can be fully mimicked by the C-terminal 11-amino acid peptide of Gtα. Furthermore, using the peptide whose leucine residues were replaced with alanine, we demonstrated that the interaction of Meta Ib with Gt was different from that of Meta II. All these results clearly showed that there are two distinct intermediate states of rhodopsin that can participate in the Gt activation process. Furthermore, the rearrangement of the C-terminal region of the Gtα during the conversion from Meta Ib to Meta II might be important for the GDP-GTP exchange reaction on Gtα.

The chromophore of Meta Ib is probably a protonated Schiff base, because the absorption maximum of Meta Ib is located at about 465 nm, which is similar to that of Meta I having a protonated Schiff base chromophore, but considerably different from that of Meta II (λ_{max} = 380 nm) having an unprotonated Schiff base chromophore. Because Meta Ib binds to Gt, as Meta II does, it is likely that deprotonation of the Schiff base chromophore after photoisomerization is unnecessary for the binding to Gt (*9*, *19*). This is consistent with the facts that invertebrate rhodopsin activates the G protein through interaction with a intermediate having a protonated Schiff base chromophore (*23*, *24*) and that the bovine rhodopsin mutant E113A/A117E, where the original counterion at position 113 is changed to position 117 (one helix turn up from the original position), forms a Gt-activating photoproduct which has a protonated Schiff base chromophore (*25*). The presence of the Gt-binding state was also detected by Fourier transform infrared (FTIR) spectroscopy, where a transient change in the amide I signal prior to the formation of Meta II was observed only in the presence of the C-terminus peptide of Gtα in the sample (*26*). These results show that deprotonation of the chromophore is unnecessary for the binding to Gt, and that there is little relationship between the deprotonation of the Schiff base chromophore and the activation of Gt. Interestingly, the mode of interaction of Meta Ib with Gt is somewhat similar to that of the Meta II intermediate of the rhodopsin mutant where the amino acids at positions 237 to 249 in the third cytoplasmic loop are deleted [Δ237–249 (*27*)]. They can bind to Gt but induce no GDP-GTP exchange reaction on Gtα. Similarly, a mutant of the β2-adrenergic receptor whose third loop region is deleted exhibits a single high-affinity agonist state but does not

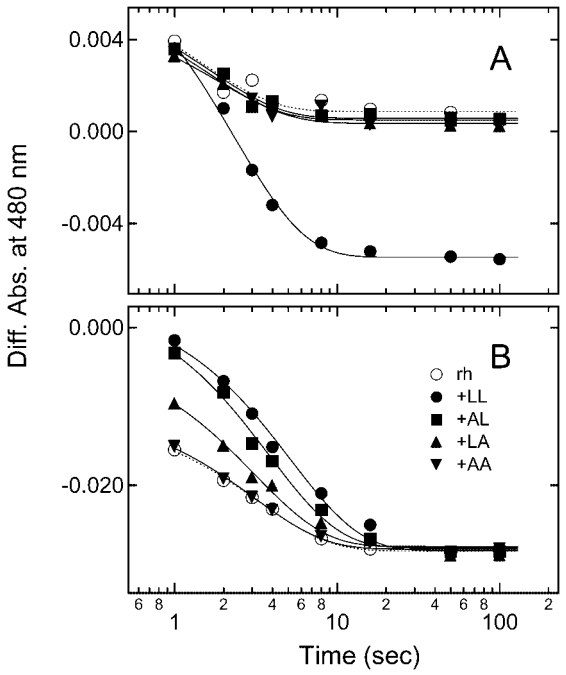

Fig. 7. **Interaction of the Gt-derived peptide and its analogues with rhodopsin.** (A and B) Time courses of absorbance changes at 480 nm during the incubation of irradiated samples containing rhodopsin (5 μM) (open circles), rhodopsin + 500 μM peptide LL (solid circles), rhodopsin + 500 μM peptide AL (solid squares), rhodopsin + 500 μM peptide LA (solid triangles), and rhodopsin + 500 μM peptide AA (inverted solid triangles), respectively. These samples contained glycerol at final concentrations of 10 (A) and 58% (v/v) (B), respectively. The samples were irradiated with >500-nm light for 1 s at 0°C, followed by incubation at this temperature for 15 min. The curves are the fitted single exponential curves with time constants of 3.4 s (open circles), 5.1 s (solid circles), 4.1 s (solid squares), 3.7 s (solid triangles), and 3.4 s (inverted solid triangles) in (A), and 1.7 s (open circles), 2.5 s (solid circles), 1.7 s (solid squares), 1.7 s (solid triangles), and 1.6 s (inverted solid triangles) in (B).

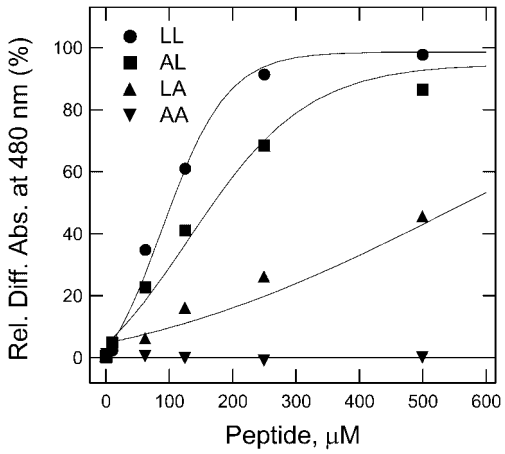

Fig. 8. **Relationship between the peptide concentration and transient trapping of Meta Ib.** The increase in absorbance at about 480 nm due to the transient trapping of Meta Ib was plotted as a function of the peptide concentration. The spectra of the samples containing only rhodopsin, rhodopsin + peptide LL (solid circles), rhodopsin + AL (solid squares), rhodopsin + LA (solid triangles), and rhodopsin + AA (inverted solid triangles) were recorded immediately after irradiation with >500-nm light for 1 s at 0°C, and the absorbance at wavelengths from 475 to 485 nm (1-nm interval) were averaged. The differences in absorbance between the samples containing peptides and the sample containing rhodopsin only were then calculated. The relative difference in absorbance at 480 nm (100%) was considered to be the amount of Meta Ib transiently trapped by a saturating amount of peptide LL (1 mM). The curves are the fitted sigmoid curves with EC50 of 90 ±5 μM (solid circles), 130 ±14 μM (solid squares), and 530 ±25 μM (solid triangles). Peptide AA had no effect on the transient trapping of Meta Ib under our experimental conditions.

activate the G-protein (*28*, *29*). Therefore, investigation of Meta Ib would provide an insight into the mechanism of the loop arrangement that is essential for activation of Gt after binding to Gt.

Although it was confirmed in the present study that Meta Ib can bind to Gt (heterotrimeric form), it should be discussed whether or not Meta Ib can bind to Gtα having GTPγS in its nucleotide binding site (Gtα-GTPγS). If Meta Ib can bind to Gtα-GTPγS, the equilibrium between Meta Ib and Meta II could shift toward Meta Ib because Meta II shows no affinity to Gtα-GTPγS. However, our present study showed no difference between the spectra of the equilibrium states obtained for the sample containing rhodopsin and that containing rhodopsin, Gt and GTPγS (Fig. 4B). That is, specific stabilization of Meta Ib by Gtα-GTPγS was not observed in our experiments. In addition, we confirmed that monomeric Gtα-GTPγS prepared according to the method previously reported (*30*) had no effect on either the decay kinetics of Meta Ib or the equilibrium between Meta Ib and Meta II (unpublished data). These results are consistent with our previous report that monomeric Gtα-GDP exhibits no affinity to either Meta Ib or Meta II (*9*). On the other hand, a synthetic peptide of the Gtα C-terminus bound to Meta Ib and Meta II (Fig. 6). This might depend on the high peptide concentration. In fact, it has been reported that the EC50 of the C-terminal peptide of Gtα is hundreds of times higher than that of the Gt trimer (*31*). Therefore, we concluded that Gtα-GTPγS has little, if any, affinity to Meta Ib, probably because of its unrestricted conformation like that of free peptides.

Nature of the Two-Step Interaction—In the present study, we addressed the roles of the two highly conserved leucine residues (L344, L349) in the C-terminal region of Gtα after confirming that the C-terminus peptide can mimic the whole Gt in the interaction with rhodopsin intermediates. Based on the observation that the replacement of one of the residues caused a loss of the ability to form extra Meta II, both L344 and L349 are necessary for the formation of a stable and functional complex with Meta II that induces a GDP/GTP exchange reaction on the nucleotide binding domain of Gtα. On the other hand, interestingly, the replacement of L349 caused a large decrease in the ability to bind to Meta Ib, whereas that of L344 only caused a slight decrease in this ability. These results suggest that L349 is more important for binding to Meta Ib than L344 is.

Based on the results of present study as well as those previously reported, we interpret the two-step interaction between rhodopsin intermediates and the C-terminal region of Gtα as follows: First, rhodopsin is converted to Meta Ib upon absorption of light and binds to Gtα

through interaction with a region near L349. Second, Meta Ib is gradually converted into Meta II, along with deprotonation of the Schiff base, and forms a stable complex. The interaction of the region near L344 with Meta II would cause the formation of the specific structure of the C-terminus that was determined by means of a NMR study (*32*). The formation of the specific structure might be essential for the GDP-GTP exchange reaction on Gtα. Recently, it was reported that the region near L344 of Gtα interacts with the third cytoplasmic loop of rhodopsin in a manner different from that of the corresponding region of Goα, by which results in different activation efficiencies (*33*). These results support our argument that the formation of a stable complex around L344 with rhodopsin would be responsible for the activation of Gt.

Through the present study together with these previously published (*9*, *19*), we have confirmed that even in a CHAPS-PC mixture, Meta II is accumulated in the presence of transducin or the C-terminal 11-amino acid peptide, and that the accumulation caused by transducin is abolished by GTPγS. These results are phenomenologically similar to those obtained with ROS as a sample. The important point is that the EC_{50} of the peptide for extra Meta II formation (90 μM) observed in this study is almost the same as that observed for ROS [100 μM; (*32*)], suggesting that the mode of interaction of Meta II with the peptide is similar under detergent (+glycerol) and ROS conditions. It has also been reported that the peptide can compete with native transducin even in a detergent containing sample (*34*). The Meta Ib-like intermediate, that is, the intermediate having a protonated Schiff base chromophore and exhibiting the ability to interact with the C-terminal peptide, was also detected in the ROS sample on FTIR spectroscopy (*26*). Overall, it is reasonable to speculate that Meta Ib is one of the physiologically relevant intermediates appearing in ROS.

We have now identified the weak binding of a rhodopsin intermediate (Meta Ib) with Gt prior to the formation of the stable complex (Meta II) at 0°C. Thus, biochemical and biophysical techniques are applicable to investigation of the physiological meaning of this two-step interaction.

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