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G-protein alpha and beta–gamma subunits interact with conformationally distinct signaling states of rhodopsin

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Abstract

Light activated rhodopsin interacts with domains on all three subunits of transducin. Two of these domains, the C-terminal regions of the alpha and gamma subunits mimic the ability of transducin to stabilize the active conformation of rhodopsin, metarhodopsin II, but display different roles in transducin activation process. Whether the interactions are with the same or different complimentary sites on Meta II is unknown. We have used chemo-selective thioalkylation of rhodopsin and UV/visible spectroscopy to show that interactions with transducin C-terminal domains can be selectively disrupted. These data provide evidence that formal structural determinants on Meta II for these domains of transducin are different. In a set of complimentary experiments we examined the reactivity of Meta II species produced in the presence of the Gtα and Gtγ subunit peptides to hydroxylamine. Analysis of the rates of Meta II decay confirms that the conformational states of Meta II when bound to Gtα and Gtβγ represent distinct signaling states of rhodopsin.

Keywords: Rhodopsin; Transducin; Alpha subunit; Beta–gamma subunit complex; Phototransduction; Signaling

1. Introduction

A prototypical seven transmembrane (7TM) domain receptor, rhodopsin, interacts with a heterotrimeric G-protein, transducin, to initiate a visual signaling cascade in response to light activation of rod photoreceptors (Hargrave, 2001; Kisselev, 2005). Despite available X-ray structures of rhodopsin and transducin, and extensive mapping of the rhodopsin–transducin interface, the exact mechanism of rhodopsin-catalyzed nucleotide exchange on transducin remains poorly understood. On rhodopsin, the site of interactions with the G-protein is comprised of cytoplasmic loops C2, C3, and a short helix in C4 (H8) (Okada, Ernst, Palczewski, & Hofmann, 2001; Park, Filipek, Wells, & Palczewski, 2004; Ridge, Abdulaev, Sousa, & Palczewski, 2003; Sakmar, Menon, Marin, & Awad, 2002). The footprint from the light-activated rhodopsin on transducin is fairly large, and includes several regions on the Gtα-, Gtβ-, and Gtγ-subunits. The C-terminal binding domains of the Gtα- and Gtγ-subunits are the major receptor recognition regions (Gautam, Downes, Yan, & Kisselev, 1998; Hamm, 2001). They are of special interest, because of the ability to mimic holo-transducin in stabilizing the active photointermediate of rhodopsin, metarhodopsin II, Meta II, or R* (Hamm et al., 1988; Kisselev, Ermolaeva, & Gautam, 1994). Both domains, Gtα(340–350), GtαCT and Gtγ(60–71)farnesyl, GtγCT have been implicated in a two-step sequential fit hypothesis, which argues for a temporal separation in interactions between Gtα and Gtβγ with R* (Herrmann et al., 2004; Kisselev, Meyer, Heck, Ernst, & Hofmann, 1999a). It has been specifically suggested that GtγCT makes initial contacts with R*, while GtαCT is involved at the second step in R*-catalyzed nucleotide exchange. Despite this information, the dynamics of GtαCT and GtγCT in the R*–Gt complex is sketchy. The question of whether light activated rhodopsin

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presents two independent sites for interactions with these domains of the Gtα- and Gtγ-subunits remains unresolved. It is also unknown whether different conformations of Meta II are involved during binding to Gtα and Gtβγ in the sequential fit hypothesis.

In order to probe the cytoplasmic surface of rhodopsin for the presence of two sites complementary to GtαCT and GtγCT, we have used chemo-selective modifications of cysteine residues at positions 140 and 316 with *N*-ethyl-maleimide (NEM) and iodoacetamidosalicilate (IAS) and their derivatives, and measured how modified R* interacts with holo-Gt in biochemical binding experiments. Both cysteines are conveniently situated in the general vicinity of the known sites for Gt on the rhodopsin cytoplasmic surface. Chemical modifications of these residues introduce potential steric hindrances at R*–Gt interface and provide an opportunity to selectively block interactions with Gtα and Gtβγ binding domains. Analysis of interactions between individual domains of R* and Gt is difficult because of strong allosteric effects in the R*–Gt complex. To circumvent these allosteric complications, we probe modified rhodopsin with synthetic peptides specific to GtαCT and GtγCT by UV/visible spectroscopy. Finally, we directly probe Meta II conformations produced in the presence of either GtαCT or GtγCT by hydroxylamine, a rhodopsin conformation-sensitive reducing agent. The Schiff base of the retinal is extremely sensitive to the attack by hydroxylamine when rhodopsin is in the Meta II state. This increased reactivity is the direct result of the light-triggered conformational changes in rhodopsin, and the opening of the transmembrane bundle. We use UV/visible and single wavelength rapid scanning spectroscopy to monitor hydroxylamine-induced Meta II decay. Our results provide support to the sequential fit hypothesis and argue that after light activation of rhodopsin, Gtα and Gtβγ interact with conformationally distinct signaling states of R*.

2. Materials and methods

2.1. Rhodopsin and Gt isolation

Dark adapted frozen bovine retinas are obtained from W.L. Lawson, Co. (NE). Rod outer segments (ROS) are prepared by the method of Papermaster and Dreyer (Papermaster & Dreyer, 1974). Urea-washed ROS membranes (UM) are prepared using the procedure adapted from Yamazaki et al. (Yamazaki, Bartucca, Ting, & Bitensky, 1982), and Willardson et al. (Willardson, Pou, Yoshida, & Bitensky, 1993), essentially as we described earlier (Kisselev, Pronin, & Gautam, 1999b). Rhodopsin concentration is measured as ΔA_{498} before and after bleaching in the presence of 20 mM hydroxylamine, based on the molar extinction coefficient at 498 nM of 42,700 $M^{-1}\,cm^{-1}$ (Hong & Hubbell, 1972). Gt was purified by GTP elution from isotonically washed ROS disks, and Gtα and Gtβγ were separated by AKTA FPLC on Blue-Sepharose Cl-6B. The purity of rhodopsin and transducin were also assessed by SDS–PAGE and protein staining with silver (Wray, Boulikas, Wray, & Hancock, 1981).

2.2. Selective double labeling of rhodopsin

Modifications at cysteine 140 with *N*-ethyl-maleimide (NEM) and at cysteine 316 by iodoacetamidosalicilate (IAS) was performed using a procedure adapted from De Grip (De Grip, Bonting, & Daemen, 1975) and Sale (Sale, Towner, & Akhtar, 1977). Dark-adapted rhodopsin in urea treated ROS membranes (1.35×10^{-4} M rhodopsin) were suspended in 0.2 M potassium phosphate buffer, pH 7.0. IAS was dissolved in a small volume of ethanol. The reaction was started by adding a 20-fold molar excess of IAS in 0.2 M potassium phosphate buffer, pH 7.0. After incubation for 18 h at 20 °C in the dark, samples were diluted by 16 reaction volumes of an ice-cold 0.2 M potassium phosphate buffer, pH 7.0, and centrifuged (4 °C, 16,000*g*, 30 min). Pellets were washed with the same buffer followed by centrifugation (4 °C, 16,000*g*, 30 min). ROS were then resuspended again at 1.35×10^{-4} M rhodopsin and further incubated with a 100-fold molar excess of [^{3}H]NEM (*N*-[ethyl-1,2-^{3}H]maleimide, specific radioactivity 1.7×10^7 dpm/μM) for 9 h at 20 °C. Samples were finally washed twice with an excess of ice cold 0.2 M potassium phosphate buffer, pH 7.0, and either processed immediately or frozen at −80 °C. For a non-selective modification of both cysteine 140 and cysteine 316 by [^{3}H]NEM, the same procedure was followed except that no IAS was added before the first 18 h incubation. The amount of rhodopsin in samples was determined both spectroscopically and using Bio-Rad Protein Assay. To confirm the site of thioalkylation reported earlier (De Grip et al., 1975; Findlay et al., 1984), aliquots of samples were treated with the endoprotease V8 (Sigma) in 50 mM ammonium bicarbonate buffer, pH 7.5, for 30 min at 37 °C (0.5 U/9 nmol of rhodopsin) to produce rhodopsin fragments Rh(1–239), Rh(240–341) and a small C-terminal fragment Rh(342–348) (Sale et al., 1977). Samples were subjected to Laemmli SDS–PAGE under non-denaturing conditions. Gels were stained with Coomassie blue R-250 and cut in 2 mm slices. The slices were placed in counting vials and incubated for 3 h at 50 °C in the presence of gel solubilizer, Solvable (NEN). After addition of the scintillation fluid, the vials were counted in a Beckman scintillation counter (model LS 1801). For UV/visible spectroscopic measurements, rhodopsin was prepared as above with non-radioactive NEM and IAS. As expected, only two cysteines were modified by NEM, Cys 140 found in the proteolytic fragment Rh(1–239), and Cys 316 found in fragment Rh(240–341), while pre-treatment with IAS selectively protected Cys 316. In preparation of spin-labeling experiments, three sulfhydryl specific derivatives of 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (PROXYL) were used, 3-iodoacetamido (IA)-PROXYL, 3-methyl-methanethiosulfonate (MTS)-PROXYL, and 3-maleimido-PROXYL (TRC Inc.).

2.3. Gt binding to ROS membranes

Various amounts of the Gtβγ (0–10 μg) were reconstituted with 10 μg of Gtα and 30 μg of urea-washed ROS membranes, UM, in 100 μl of buffer ROS-ISO (10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.1 mM PMSF) on ice. The reaction was initiated by exposure to light. UM were centrifuged at 109,000*g*, 4 °C for 10 min in a TLA-100.3 rotor on a Beckman TL-100 Ultracentrifuge. The pellet was washed twice with buffer ROS-ISO. UM with Gt bound was resuspended in 50 μl of buffer 10 mM Tris–HCl, pH 7.4, 0.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and 250 μM GTPγS, incubated on ice for 30 min, and centrifuged. The supernatant was analyzed for the presence of G-protein subunits by immunoblotting. Results were quantified with Image Gauge (FujiFilm). Data analysis and curve fitting was in KaleidaGraph 3.6.2.

2.4. Peptides

Peptide synthesis, purification, and mass-spectrometry were as we described before (Kisselev et al., 1994). Pure peptides were stored at −85 °C, lyophilized and under nitrogen. Before the experiments the peptides were dissolved in water to obtain stock solutions of 1–10 mM, and the pH was adjusted to 7.5 with NaOH. Amino acid sequences for the peptides were as follows: GtαCT–Gtα(340–350) (IKENLKDCGLF), GtγCT—Gtγ(50–71) farnesyl (EDPLVKGIPEDKNPFKELKGGC-farnesyl), which is functionally equivalent to Gtγ(60–71)farnesyl but gives more complete Meta II stabilization.

2.5. UV/visible spectroscopy

The amount of extra Meta II was measured on a Cary-50 UV/visible spectrophotometer (Varian, CA), at 4 °C, cuvette path-length 10 mm,

essentially as we described before (Kisselev et al., 1994; Kisselev et al., 1999a). Samples contained 2.5 μM of urea-washed ROS membranes, or an equal amount of the rhodopsin membranes treated with NEM and/or IAS in buffer MII (20 mM Tris–HCl, pH 8.0, 130 mM NaCl, 1 mM $MgCl_2$, and 1 mM EDTA) and 2 mM of corresponding G-protein peptides. 700–250 nm spectra were recorded before and after activation of the sample with a 390 ± 5 nm light. The amount of Meta II was calculated as the absorbance difference A380–A417 before and after photoactivation. The amount of Meta II without transducin peptides added was taken as zero. Control Meta II experiments were at pH 5.5 in 80 mM 2-[*N*-Morpholino]-ethanesulfonic acid, MES, pH 5.5, 130 mM NaCl, 1 mM $MgCl_2$, and 1 mM EDTA. Single wavelength experiments were as above, except that they utilized Cary-50's rapid scanning mode at 80 data points/s. Buffered hydroxylamine was added to the cuvette to the final concentration of 50 mM in the dark (Hofmann, Emeis, & Schnetkamp, 1983; Sakmar, Franke, & Khorana, 1991). The data were collected at 365, 405, and 500 nm in separate experiments. The total length of each scan was 10 min. No noticeable bleaching of the sample was detected due to the Cary-50 scanning beam either with or without hydroxylamine. The sample was activated halfway through the scan with a shutter-controlled Fiber-Light PL-900 equipped with a 390 ± 5 nm pass filter. The data were processed off-line using KaleidaGraph 3.6.2. Full spectra scans were normalized to zero at 700 nm. Single wavelength scans were normalized to zero using baseline absorbance values immediately before light activation. For the analysis of rates of Meta II decay, the traces were normalized to the peak maximum of Meta II.

3. Results

3.1. Chemo-selective thioalkylation of rhodopsin

We used selective thioalkylation of dark-adapted rhodopsin in native urea-washed rod outer segment membranes in order to introduce potential steric hindrances for interactions between R* and binding domains GtαCT and GtγCT. We used a rhodopsin modification procedure originally described by Wu and Stryer (Wu & Stryer, 1972) and by De Grip (De Grip et al., 1975), which takes advantage of the ability of *N*-ethyl-maleimide (NEM) to modify preferentially two surface cysteine residues under mild reaction conditions in the dark, while treatment with another agent, iodoacetamidosalicilate (IAS), leads to the selective modification of a single residue. After the discovery of the full amino acid sequence of bovine rhodopsin (Hargrave et al., 1983; Ovchinnikov et al., 1982), these cysteine residues have been identified as Cys 140 and Cys 316 at the cytoplasmic surface of rhodopsin, Fig. 1. Using [^{3}H]NEM we confirmed the reported chemo-selectivity of NEM and IAS by monitoring the incorporation of [^{3}H]NEM into rhodopsin with and without pre-treatment with IAS (Methods). Based on this procedure, three samples were prepared: (1) R-IAS, IAS-treated rhodopsin with Cys 316 modified, (2) R-NEM, NEM-treated rhodopsin with both Cys 140 and Cys 316 modified, and (3) R-IAS/NEM, rhodopsin treated with IAS to modify Cys 316, and then treated with NEM to modify remaining Cys 140.

3.2. Photoactivation of the modified rhodopsin

First, we studied whether the chemo-selective thioalkylation of rhodopsin would affect Meta II formation under low pH conditions where Meta II is a predominant spectral intermediate. All three modified samples were indistinguishable from native rhodopsin in full dark–light UV/visible scans, Fig. 2. This experiment confirms a previous report (Resek, Farahbakhsh, Hubbell, & Khorana, 1993) that mild thioalkylation at Cys 140 and Cys 316 at the rhodopsin's cytoplasmic surface does not interfere with Meta II formation.

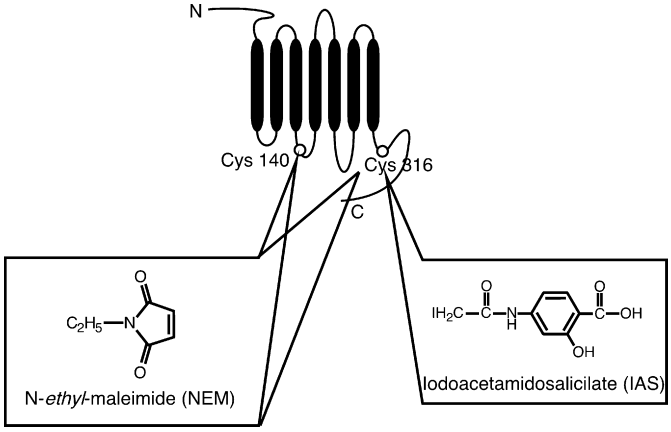

Fig. 1. A schematic presentation of two sites of chemo-selective thioalkylation of rhodopsin.

3.3. Stabilization of extra-Meta II

Under more basic pH 8.0, light activation of rhodopsin produces Meta I–Meta II equilibrium that is significantly shifted towards Meta I. Addition of synthetic peptides that represent GtαCT and GtγCT leads to extra-Meta II in a

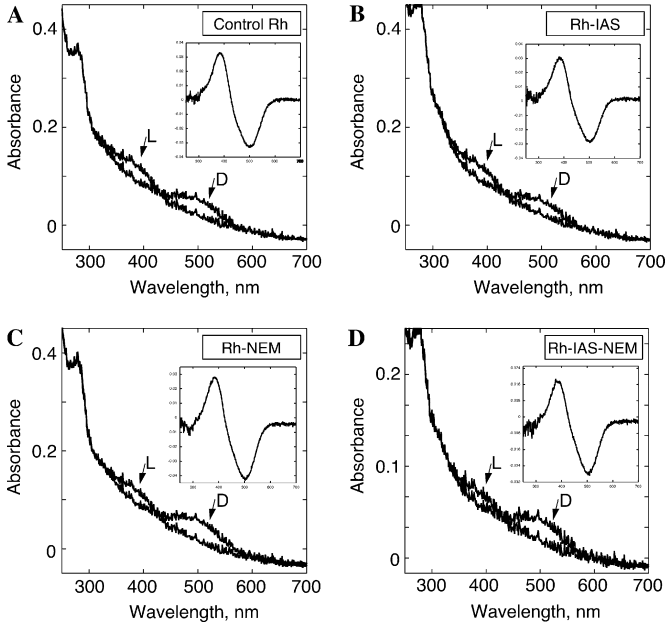

Fig. 2. UV/visible spectra of urea-washed ROS membranes. The light minus dark difference spectra are shown in insets. (A) The native rhodopsin. (B) Rhodopsin modified with IAS at Cys 316. (C) Rhodopsin modified with NEM at Cys 140 and Cys 316. (D) Double modification with IAS at Cys 316 and subsequently with NEM at Cys 140.

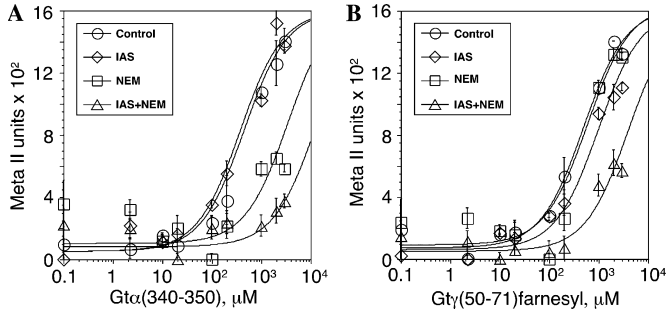

Fig. 3. Stabilization of extra Meta II by GtαCT, Gtα(340–350) (A), and GtγCT, Gtγ(50–71)farnesyl (B). The type of the thioalkylating agent used for rhodopsin modification is shown with different symbols. Error bars represent standard deviation calculated from at least three independent experiments.

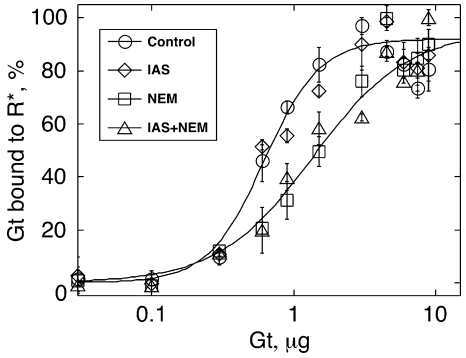

Fig. 4. Dose-dependent interaction of Gt with urea-washed ROS membranes. The type of the thioalkylating agent used for rhodopsin modification is shown with different symbols. Error bars represent standard deviation calculated from four independent experiments.

dose-dependent manner, an effect that mimics holo-Gt. We used this spectroscopic assay to measure whether selective modifications of Cys 140 and Cys 316 in rhodopsin would interfere with extra-Meta II signal. R-IAS showed dose-dependent Meta II formation in the presence of GtαCT indistinguishable from native R, with $EC_{50} = 300\,\mu M$. In striking contrast, R-NEM and R-IAS-NEM were unable to interact with GtαCT effectively, showing at least a 10-fold reduction in affinity, $EC_{50} = 3\,mM$ and $EC_{50} > 10\,mM$ correspondingly, Fig. 3A. Because all rhodopsin samples showed normal dark spectra and normal Meta II formation at pH 5.5, the loss of extra-Meta II formation was not due to the partial inactivation of rhodopsin by NEM, or IAS and NEM.

Extra-Meta II measurements utilizing GtγCT produced a distinctly different pattern of interaction. Contrary to the lack of effect on GtαCT binding, modifications of rhodopsin with IAS produced a 3-fold decrease in GtγCT affinity for R*, Fig. 3B. NEM had no effect on Meta II stabilization by GtγCT, while double modification, Cys 316 with IAS and subsequent modification of Cys 140 with NEM increased EC_{50} to 4 mM. Similar extra-Meta II data were obtained when pyrrolidine–nitroxide-based spin labeling reagents (IA)-PROXYL were used instead of IAS, and (MTS)-PROXYL and 3-maleimido-PROXYL were used instead of NEM. Thus, modifications of rhodopsin's Cys 140 and Cys 316 had an almost opposite effect on the ability of GtαCT and GtγCT to interact with Meta II. The same rhodopsin sample modified by NEM interacted with GtγCT normally, but with GtαCT poorly. Rhodopsin modified by IAS, in contrast, interacted with GtαCT well, while GtγCT binding to Meta II was reduced.

3.4. Interactions with Gt

To study how the chemo-selective thioalkylation of rhodopsin would affect light-dependent binding of holo-Gt to R, R-IAS, R-NEM, and R-IAS-NEM, we independently reconstituted these rhodopsin samples with increasing amounts of Gt made from purified Gtα and Gtβγ. Only GDP retained on Gtα after Gt purification was present in the assay. The rhodopsin membranes were illuminated, washed, and then the bound Gt was examined in GTPγS eluates by quantitative immuno-blotting, Fig. 4. Gt binding to native membranes exhibited clear signs of positive cooperative interaction, also reported earlier by Willardson (Willardson et al., 1993). Fitting the Gt binding data to the Hill equation revealed Hill coefficient 2.2 for R*–Gt interaction. When Cys 316 of rhodopsin was modified by IAS, we observed no change in the binding characteristics of Gt. When both Cys 140 and Cys 316 were modified by NEM, or IAS and NEM combination, the total level of Gt binding at high Gt concentrations did not change, which is consistent with a previous report (Reichert & Hofmann, 1984), but Gt binding lost positive cooperativity, Hill coefficient is 1.3.

3.5. Meta II decay induced by hydroxylamine

In the second set of experiments we directly examined the conformational status of Meta II produced by GtαCT or GtγCT in the presence of hydroxylamine. Just as described above, after illumination under pH 8.0 and 4 °C, Meta I, $A_{max} = 478\,nm$, is the predominant photoproduct, while in the presence of synthetic peptides GtαCT and GtγCT, Meta II predominates, $A_{max} = 380\,nm$, Fig. 5A and B. In the presence of 50 mM hydroxylamine dark rhodopsin is very stable. Addition of GtαCT or GtγCT at 2 mM did not change dark rhodopsin stability in the presence hydroxylamine, as monitored by the absorbance change at 500 nm, showing that peptides did not influence the Schiff base accessibility, Fig. 5C. Only after light activation can hydroxylamine attack the Schiff base to form retinal oxime, $A_{max} = 365\,nm$, Fig. 5B (L + HA) (Hofmann et al., 1983; Sakmar et al., 1991). We monitored the rate of the hydroxylamine induced Meta II decay, when Meta II was produced by GtαCT or GtγCT. Because of the spectral overlap between Meta II and retinal oxime peaks, Fig. 5B, we measured Meta II decay using rapid scanning spectroscopy at 405 nm. Under these conditions, light activation of the sample leads to the fast and transient formation of Meta II, which is then converted to the retinal oxime by the present

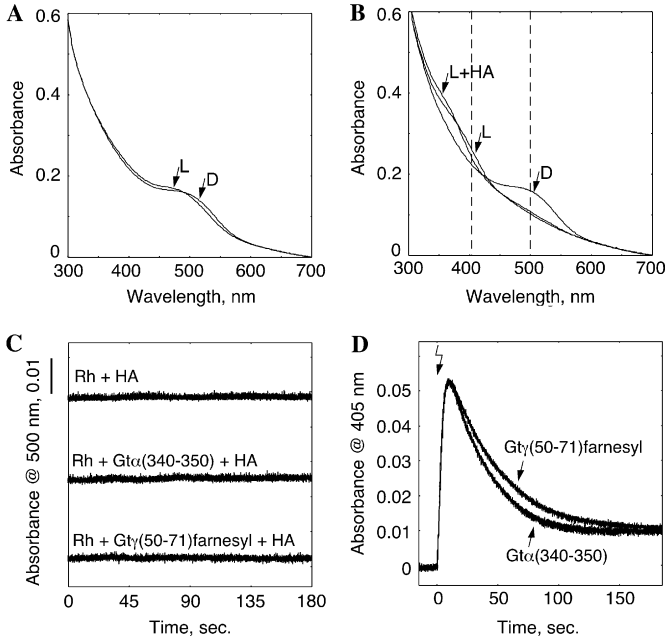

Fig. 5. Reactivity of Meta II–GtαCT and Meta II–GtγCT to hydroxylamine. (A) Control UV/visible spectra of rhodopsin membranes at pH 8.0. (B) Representative UV/visible spectra of rhodopsin membranes in the presence of GtαCT in the dark (D), after light activation (L), and after subsequent addition of hydroxylamine (L + HA). Dotted lines show wavelengths used to collect time resolved data at 500 and 405 nm. (C) Time resolved spectra of dark rhodopsin in the presence of hydroxylamine at 500 nm (Rh + HA), and after addition of GtαCT, Gtα(340–350) (Rh + Gtα(340–350 + HA)), and GtγCT, Gtγ(50–71)farnesyl (Rh+ Gtγ(50–71)farnesyl + HA). (D) Transient formation of Meta II and hydroxylamine-induced decay. The arrow indicates the start of light activation.

hydroxylamine, Fig. 5D. The initial rate and time to peak of Meta II production were identical for both GtαCT and GtγCT. Comparison of the rates of Meta II decay revealed that Meta II–GtγCT decays substantially slower than Meta II–GtαCT. The rate of Meta II–GtαCT decay was identical to the rate of Meta II decay without the peptides. We proceeded to measure how specific the effect of GtγCT was. The unfarnesylated GtγCT with identical amino acid sequence does not interact with Meta II (Kisselev et al., 1994). This control peptide produced the profile of rhodopsin bleaching in the presence of hydroxylamine indistinguishable from that of Meta II or Meta II–GtαCT (data not shown). Thus, specific complex Meta II–GtγCT reacts with hydroxylamine slower than Meta II–GtαCT.

4. Discussion

Rhodopsin-catalyzed nucleotide exchange on Gt is a complex multi-step reaction that involves several intermediate steps (Chabre & le Maire, 2005; Okada et al., 2001; Park et al., 2004; Ridge et al., 2003; Sakmar et al., 2002), multiple rhodopsin intermediates (Arnis & Hofmann, 1993; Morizumi, Imai, & Shichida, 2005) and specific domains on all subunits of Gt (Gautam et al., 1998; Hamm, 2001). Several models exist to explain the mechanism of R*-catalyzed G-protein activation. The lever-arm model (Iiri, Farfel, & Bourne, 1998) and the gear-shift model (Cherfils & Chabre, 2003) assume interactions of Gt with R* monomer, and share the description of the first step, the initial docking of Gt to R*, but differ in explaining the mechanism of the nucleotide release on Gtα. In short, the activating signal from R* is proposed to propagate via well-defined sites on Gtα, such as the N-terminal helix, the C-terminal domain, the α5-helix, and also via the C-terminal domain of Gtγ, shown by arrows 1 and 2 on Fig. 6. The end-result is the pulling motion by Gtβγ on the Switch II region of Gtα and release of GDP. This view has originally been proposed by Bourne and is based on the analysis showing that Gtβγ occupies the space of a nucleotide exchange factor for the Gtα-subunit, similar to the position of a nucleotide exchange factor EF-Ts, crystallized in complex with the nucleotide-empty elongation factor Tu (Ef-Tu) (Wang, Jiang, Meyering-Voss, Sprinzl, & Sigler, 1997). The gear-shift model incorporates this step as the engagement of the second gear, potentially necessary for the stabilization of the transient nucleotide-empty state, but it does not believe that this step is responsible for the nucleotide exchange. The gear-shift model proposes the engagement of the third gear, the third route, shown by arrow 3 on Fig. 6, and argues that a pushing motion by the N-terminal domain of Gtγ partially displaces the helical domain of Gt and triggers GDP release.

A conceptually different model of interactions between Gt and rhodopsin dimer has been proposed recently by

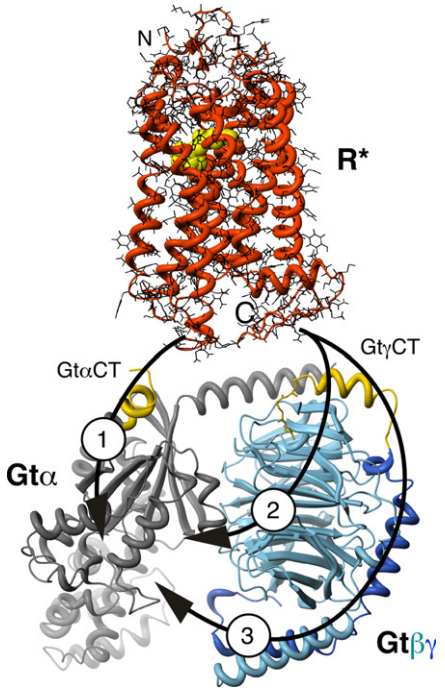

Fig. 6. Three-dimensional structures of rhodopsin (orange) and the G-protein, transducin (grey—Gtα, blue—Gtβγ). Rhodopsin interacting domains of Gt at the C-terminal regions of Gtα and Gtγ are shown in yellow in their R*-bound conformations (Kisselev & Downs, 2003; Kisselev et al., 1998). Arrows indicate proposed mechanisms of signal propagation from R* to the nucleotide-binding site of Gtα.

Palczewski (Filipek et al., 2004; Palczewski, 2006; Park et al., 2004), based on the original observation of rhodopsin dimers in the native disk membranes (Fotiadis et al., 2003). The most important feature of this model is the almost perfect match in size between RR and Gt, which explains the paradox between the large footprint on all three subunits of Gt from a relatively compact intracellular surface of R*.

In analyzing the contributions by Gtα and Gtβγ into the first step of these models, we have previously proposed a two-step sequential fit model of R*–Gt interactions to explain the mechanism of signal transfer from Meta II to Gt via the C-terminal domains of Gtα (GtαCT) and Gtγ (GtγCT) (Herrmann et al., 2004; Kisselev et al., 1999a). The model is based on the observation that both GtαCT and GtγCT mimic holo-Gt in the ability to interact with and stabilize Meta II, but they play different roles in the activation process (Herrmann et al., 2004; Kisselev et al., 1999a). The differences can potentially be explained by interactions with multiple iso-spectral forms of Meta II, or structurally unique complementary binding sites on Meta II.

In this study, we show that chemo-selective modification of rhodopsin's Cys 140 leads to a more than 10-fold reduction of GtαCT interactions with Meta II, while the modification of Cys 316 is inconsequential for GtαCT binding. The pattern of interactions is reversed when GtγCT is used to stabilize Meta II. GtγCT tolerates the modification of Cys 140 very well, but its affinity for Meta II is reduced 3-fold when Cys 316 is modified, Fig. 3. It is important to note that the identification of Cys 316's involvement in the GtγCT binding in this study, gives an important clue as to the location of the site of GtγCT and Gtβγ interactions with rhodopsin, the helix H8. This observation is consistent with a previous report showing that replacement of the amino acid sequence of H8 with the corresponding sequence from the β2-adrenergic receptor abolishes interactions with GtγCT, as well as with GtαCT (Ernst et al., 2000). Our data additionally support the models suggesting that Cys 140 is part of the binding site for GtαCT (Arimoto, Kisselev, Makara, & Marshall, 2001; Janz & Farrens, 2004). With regard to the nature of the binding sites and their involvement in the Gt activation process, the data presented here clearly demonstrate that while the binding sites for GtαCT and GtγCT partially overlap (Ernst et al., 2000; Herrmann et al., 2004), they are distinct enough to allow selective disruption of either Meta II–GtαCT or Meta II–GtγCT interactions. Thus, during the activation process rhodopsin presents structurally distinct sites for the interactions with Gtα and Gtβγ subunits, which argues for structural heterogeneity of Meta II species.

The two putative conformations of Meta II can exist in dynamic equilibrium, but they cannot exist at the same time, which argues strongly for the temporal separation of interactions between Meta II–GtαCT and Meta II–GtγCT. Although Gt mimetic peptides have proven to be powerful tools in dissecting mechanism of signal transfer from R* to Gt, there are obvious limitations of this approach because it restricts analysis to interactions of individual domains. It would be extremely interesting to extend the studies reported herein to the analysis of the domain interactions in R*–Gt complex.

The thioalkylation of Cys 140 had an interesting effect on dose-dependent Gt binding to the urea-washed ROS membranes. While the maximal level of binding was not affected, the cooperative nature of interactions was abolished, Fig. 4. One possible conclusion from this experiment is that allosteric interactions of Gtα and Gtγ with R* contribute to the positive cooperativity of binding. Exclusion of GtαCT from interactions leads to the loss of the cooperative effect. A somewhat unexpected observation is that by preventing GtαCT interactions with R*, binding of Gt and its nucleotide dependent release is not completely eliminated, Fig. 4. Perhaps Gtα can accommodate the loss of GtαCT–rhodopsin interaction through the involvement of multiple domains.

To probe Meta II–GtαCT and Meta II–GtγCT conformations, we used a well-known selective susceptibility of Meta II to hydroxylamine (Hofmann et al., 1983; Sakmar et al., 1991). In the dark state, the organization of rhodopsin's transmembrane helices protects the Schiff base from the attack by hydroxylamine, but after the light activation the reactivity to hydroxylamine is dramatically increased. We show that the rate of Meta II decay to retinal oxime is noticeably slower for Meta II–GtγCT than for Meta II–GtαCT. Because Meta II is the dominant photoproduct under the experimental conditions, the rate difference of Meta II decay argues for the differential reactivity of the Schiff base in Meta II–GtαCT and Meta II–GtγCT to hydroxylamine. These data strongly support the conclusion from the chemo-selective thioalkylation experiments above, that Meta II–GtαCT and Meta II–GtγCT are structurally distinct.

In conclusion, we show that the sites of interactions for the C-terminal domains of Gtα and Gtγ subunits on Meta II are distinctly different. It is possible to affect one site but not the other by the chemo-selective thioalkylation of rhodopsin. In addition we show that the rates of hydroxylamine-induced Meta II decay are different, depending on whether the peptides from Gtα or Gtγ were used to stabilize Meta II. Together, our results argue for the conformational and structural heterogeneity of the active photointermediate Meta II, and provide strong support to the two-step sequential fit model, under which Gtα and Gtβγ interact with unique signaling states of light-activated rhodopsin.

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