Conformational Dynamics of Activation for the Pentameric Complex of Dimeric G Protein-Coupled Receptor and Heterotrimeric G Protein

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DOI 10.1016/j.str.2012.03.017

SUMMARY

Photoactivation of rhodopsin (Rho), a G protein-coupled receptor, causes conformational changes that provide a specific binding site for the rod G protein, Gt. In this work we employed structural mass spectrometry techniques to elucidate the structural changes accompanying transition of ground state Rho to photoactivated Rho (Rho*) and in the pentameric complex between dimeric Rho* and heterotrimeric Gt. Observed differences in hydroxyl radical labeling and deuterium uptake between Rho* and the (Rho*)2-Gt complex suggest that photoactivation causes structural relaxation of Rho following its initial tightening upon Gt coupling. In contrast, nucleotide-free Gt in the complex is significantly more accessible to deuterium uptake allowing it to accept GTP and mediating complex dissociation. Thus, we provide direct evidence that in the critical step of signal amplification, Rho* and Gt exhibit dissimilar conformational changes when they are coupled in the (Rho*)2-Gt complex.

INTRODUCTION

Rho (M, 42 kDa, 348 amino acids), a prototypical G protein-coupled receptor (GPCR), is activated by light (Palczewski, 2006). This photoactivation involves isomerization of Rho’s covalently bound ligand, 11-cis-retinal, to the all-trans conformation and promotes structural changes leading to the binding of the G protein, transducin (Gt, M, 85.6 kDa, 764 amino acids) required for signal amplification (Jastrzebska et al., 2010, 2011a). Elucidation of X-ray crystal structures of Rho and several Rho photointermediates have greatly increased our understanding of the structural rearrangements produced by photoactivation (Choe et al., 2011; Deubi et al., 2012; Higgins et al., 2006; Lodowski et al., 2007; Nakamichi and Okada, 2006a, 2006b, 2007; Okada, 2004; Okada et al., 2004; Palczewski et al., 2000; Salom et al., 2006a, 2006b; Scheerer et al., 2008; Standfuss et al., 2007, 2011; Stenkamp, 2008; Teller et al., 2001). However, the Meta II state of Rho (Rho*) is transient, and crystals of Rho* provide only low-resolution data because of crystal packing alterations (Salom et al., 2006a, 2006b). The crystal structure of opsin with bound Gt peptide at low pH, postulated to represent an active conformation of Rho*, does show light-induced structural reorganization, such as an elongation of the protein (Scheerer et al., 2008), but opsin does not contain chromophore and its activity is much lower than that of Rho* (Choe et al., 2011; Scheerer et al., 2008). Crystals of opsin soaked with all-trans-retinal at low pH exhibit some properties of Rho*, but this procedure did not cause transition to ground state Rho when 11-cis-retinal was used (Choe et al., 2011), suggesting that crystal packing of opsin interferes with necessary conformational rearrangements to the native-like state (Figure 1). Therefore, the property of Rho* that enables binding to Gt, and more importantly the structure of Rho* in complex with Gt, mediates signal transduction, has yet to be determined.

Membrane proteins, including Rho, employ internal water molecules to provide a flexible link between allosteric sites (Meyer, 1992). Yet, there are few available methods that can monitor dynamic changes in reorganization of water molecules—most notable is radiolytic footprinting, with hydroxyl radical labeling and mass spectrometry readout, which is emerging as a novel approach for assessing dynamics of water (Adare et al., 2011; Angel et al., 2009a, 2009b; Orban et al., 2010). As shown previously, Rho and other receptors in the GPCR family contain conserved internal water molecules involved in mediation of the activation signal across membranes (Angel et al., 2009a, 2009b). Long molecular dynamics simulations (1.5 μs) and 1H magnetic angle spin nuclear magnetic resonance (NMR) both indicated that increased hydration accompanies development of Rho* and that subtle changes in internal hydration may be important during activation (Hurst et al., 2010). This water plays a structural role in receptor activation similar to well-recognized prosthetic groups in other proteins. In hydroxyl radical footprinting experiments, significant changes in the rates of water mediated residue modifications in Rho upon photoactivation and transition to Rho* were observed close to the chromophore binding pocket and toward the cytoplasmic face of the receptor that is important for Gt binding (Angel et al., 2009b). However, it is currently unknown if further reorganization of internal water molecules occurs as the Rho*–Gt complex develops, which would indicate both
Structure

Dynamics of Rhodopsin-Transducin Complex

![Diagram of rhodopsin activation](Image)

2006a), Opsin, the chromophore-free form of Rho, adopts an active G\textsubscript{t} binding state at low pH, termed ops\textsuperscript{*}\textsubscript{t} (Scheerer et al., 2008). Soaking ops\textsuperscript{*}\textsubscript{t} crystals (PDB entry 3dqb [Scheerer et al., 2008]) with all-trans-retinal at low pH resulted in reversible formation of the Rho\textsuperscript{*}\textsubscript{t}-like state with (PDB entry 3pqr [Choe et al., 2011]) and without the G\textsubscript{t}, C-terminal peptide (PDB entry 3pxo [Choe et al., 2011]). In the presence of G\textsubscript{t} (transducin) Rho\textsuperscript{*} forms a Rho-G\textsubscript{t} complex, but details about this structure are as yet unknown. Gathering structural information about the Rho\textsuperscript{-G\textsubscript{t}} complex was approached indirectly from two different perspectives—the first started with the Rho state and the second started with the ops\textsuperscript{*} state. The only experimentally available structural model for the Rho\textsuperscript{*}\textsubscript{t}-G\textsubscript{t} complex is a recently described low-resolution electron microscopic model (Jastrzebska et al., 2011).

a structural and functional differences in conformation between Rho\textsuperscript{*} and Rho\textsuperscript{t} complexed to G\textsubscript{t}.

GPCRs form oligomeric structures in membranes (Park et al., 2008). Consistent with these findings and using nucleotide depletion, we have extracted the Rho\textsuperscript{*}\textsubscript{t}-G\textsubscript{t} complex from native biological membranes (Jastrzebska et al., 2009) or reconstituted the complex from purified native proteins and affinity chromatography (Jastrzebska et al., 2011c). The resulting nucleotide-free Rho\textsuperscript{*}\textsubscript{t}-G\textsubscript{t} complex was composed of (Rho)\textsubscript{2}-G\textsubscript{t}, because its mass determined by scanning transmission electron microscopy was 221 ± 2 kDa and the molecular envelope calculated at 21.6 Å from projections of negatively stained protein particles demonstrated a complex structure composed of two Rho molecules together with one G\textsubscript{t} heterotrimer (Jastrzebska et al., 2011c). This provides a substrate upon which to assess structural and dynamics questions of G protein signaling in the context of a native-like assembly of the relevant macromolecular complexes.

The recent crystal structure of the β\textsubscript{2}-adrenergic receptor (β\textsubscript{2}-AR) in complex with G\textsubscript{s} has provided the highest resolution snapshot of the interaction between a monomeric GPCR and its cognate G protein (Rasmussen et al., 2011). Formation of this complex involved substantial structural changes in both proteins, including, among others, outward movements of transmembrane helices TM-V and TM-VI of β\textsubscript{2}-AR and a remarkably large displacement of the helical domain as well as rearrangements in the α subunit of G\textsubscript{s}. This landmark achievement offers invaluable insight into the mechanism of signal transduction by a GPCR across the cell membrane. However, the addition of a T4 lysozyme molecule to the amino terminus of β\textsubscript{2}-AR and the presence of a single-domain antibody necessary for stabilization of the complex may block other important dynamics necessary to the native activation mechanism. To take full advantage of this valuable static view of a GPCR bound to its G protein, application of experimental techniques capable of capturing the behavior and dynamics of the complex in solution is critical. Thus, recent studies using hydrogen-deuterium exchange experiments that characterized the dynamics of the β\textsubscript{2}-AR alone (Zhang et al., 2010) and in the presence of ligands (West et al., 2011) are quite valuable. The dynamics of G\textsubscript{s} protein induced by β\textsubscript{2}-AR was also studied (Chung et al., 2011), where presumably 1:1 complex between the receptor and G protein was formed.

In the current study, we employed structural MS, including radiolytic footprinting and hydrogen-deuterium exchange, to investigate dynamic differences in the Rho structure that occur upon photoactivation and binding to G\textsubscript{s}. Both methods are very well suited for the measure of solvent accessibility. However, there are some notable differences that make their use not redundant but rather complementary. First, the timescale for radiolytic footprinting is on the order of ms, whereas the timescale for the hydrogen-deuterium exchange is on the s or min timescale. Second, radiolytic footprinting was recently shown to be a unique tool to study internal water dynamics. Our study also investigated the dynamics of a GPCR, including its internal water molecules, induced by its G protein binding together with an examination of the dynamics of G\textsubscript{s} induced by dimeric Rho*. This assessment of the pentameric complex is compared to existing structural and dynamic data to extend models of G protein activation and dynamics.

RESULTS

Functional Characterization of Rho, Rho*, and Rho*+G\textsubscript{t} Preparations

To visualize dynamic changes in Rho upon activation and binding its heterotrimeric G protein (G\textsubscript{t}) by proteomics methods we used well-characterized preparations composed of high-purity-protein components with defined stoichiometry that retained appropriate enzymatic activities (see Experimental Procedures). Rho was purified by Zn\textsubscript{2+}-opsin extraction and de- lipidated by succinylated Concanavalin A (sConA) affinity chromatography. The native-like (Rho)\textsubscript{2}-G\textsubscript{t} complex was isolated by sConA affinity chromatography as recently described (Jastrzebska et al., 2011c). The complex was formed only after Rho was photoactivated, and it could be eluted from the column with α-methyl-D-mannoside. (Rho)\textsubscript{2}-G\textsubscript{t} dissociated to individual subunits when 200 μM GTP\textsubscript{y}S was added, indicating that the complex retained its native-like biochemical activity. The purified complex exhibited an absorbance maximum at 380 nm and retinoid analysis showed that the major chromophore isomer
was all-trans-retinal as expected for Rho*. Bound to Rho*, Gt was free of nucleotides based on high-performance liquid chromatography (HPLC) nucleotide analysis. After X-ray irradiation for footprinting analysis, spectral properties of Rho and the Rho*-Gt complex did not exhibit major changes and gel analysis did not show a significant amount of amide bond cleavage as previously reported (Angel et al., 2009b). Individual proteins and the complex were subjected to radiolytic footprinting (Adare et al., 2011; Angel et al., 2009a, 2009b; Orban et al., 2010b) and hydrogen-deuterium exchange (Busenlehner and Armstrong, 2005); next, proteins were cleaved proteolytically, and the generated peptidic fragments were identified and quantified by MS. Control experiments involved the use of synthetic peptides.

Optimization of Proteolysis of Rho and Gtα with Pepsin and Peptide Separation Yielded High Coverage for Both Proteins

Successful rapid digestion and peptide separation of proteins with multiple TM domains is still challenging, and optimization of digestion conditions is essential (Konermann et al., 2011). We found that the 10 min digestion of Rho and Gtα with pepsin for the hydrogen-deuterium exchange experiments resulted in an excellent sequence coverage ranging from 83%–90% for Rho and 82%–87% for the alpha subunit of Gt. For overnight pepsin digests of Rho employed for radiolytic footprinting studies, the sequence coverage was even higher (typically >95%). Because we employed highly purified proteins, the spectra were devoid of extraneous peptides. The quality of identification was based on three independent scoring models—two statistical and one descriptive. Based on parameters described in detail elsewhere, all peptides we considered identified had pp or pp2 scores higher than six, indicating statistical significance (Xu and Freitas, 2009a).

Binding of Gt to Rho* Changed Internal Water Reactivity as Determined by Radiolytic Footprinting

To identify changes in internal waters reorganization that resulted from Rho photoactivation and the complex formation with Gtα, we used X-ray-induced radiolytic footprinting. In this hydroxyl radical labeling approach, we identified eighteen peptide fragments that were modified after exposure to high-flux X-rays (Figure 2A). These peptides encompassed fragments of the sequence derived from TM-VI (W35, M44, M49, P71, M86, A117, A124, M143, M155, M163, M253, P285, M288, P303, and M317), the plug region of the chromophore (M183 and P194), and the C-terminal region (A346). These modifications were consistently identified in all preparations. Scoring functions used for identification were identical for both modified and unmodified peptides. Dose response plots were constructed for five peptide fragments.
fragments that provided reproducible and reliable quantification for all three states of Rho examined (Figures 3A–3E) and one for a peptide fragment identified only in the case of the Rho*-G complex (Figure 3F). The modification rate of the C-terminal peptide fragment denoted as ASTTVSKTETSQVA\textsuperscript{346}PA was the highest in the Rho*-G complex. Fragments identified as FM\textsuperscript{86}VFGGF, FAKTSAVYNP\textsuperscript{303}VIY, and M\textsuperscript{288}TIPAF showed significant differences in oxidation rate between Rho*-G complex as compared with Rho and Rho* (Figures 3B, 3D, and 3E) although the error of the measurements was higher for these peptides as compared to the peptide fragment ASTTVSKTETSAPA. Modification rates for the TWV\textsuperscript{163}MA fragment, although high, showed no difference between the three states of Rho (Figure 3C). The changes in rates can be attributed to reorganization of locally trapped waters within the core of the protein (Angel et al., 2009b; Orban et al., 2010b).

**Figure 3. Radiolytic Footprinting of Rho, Rho*, and Rho*-G**

Dose plots showing fraction unmodified (see the Experimental Procedures subsection “Radiolytic Footprinting”) as a function of exposure time (ms) were constructed for six peptides from a total of 18 that showed modification after exposure to X-rays (see Figure 2A, in red). These were analyzed as described in the Experimental Procedures subsection “Radiolytic Footprinting.” (A) Shows fragment ASTTVSKTETSQVA\textsuperscript{346}PA, (B) shows fragment FM\textsuperscript{86}VFGGF, (C) shows fragment TWV\textsuperscript{163}MA, (D) shows fragment FAKTSAVYNP\textsuperscript{303}VIY, (E) shows fragment M\textsuperscript{288}TIPAF, and (F) shows fragment SATTQKAKEVTTRM\textsuperscript{335}. In all panels peptide fragments obtained from Rho samples are indicated by black squares, Rho* by red spheres, and Rho*-G by blue triangles. Error bars represent SEM, and statistical significance was assessed by the Student’s t test. Data represent at least three independent experiments.
Insight into Rho* and Gt Dynamics from Hydrogen-Deuterium Exchange

Hydrogen-deuterium exchange coupled to MS is a powerful method to study protein dynamics (Busenlehner and Armstrong, 2005; Konermann et al., 2011). Each hydrogen-deuterium experiment was carried out following the same workflow (Figure 4). A primary concern after quenching the exchange reaction is to minimize back exchange of deuterium to hydrogen during the

Figure 4. Typical Flowchart of the Hydrogen-Deuterium Exchange Analysis
(A) Shows a typical chromatogram depicting relative intensities (%) of the signal as a function of elution time (min) obtained after the digestion of Rho with pepsin (details are described in the Experimental Procedures subsection “Amide Hydrogen-Deuterium Exchange of Rho”).
(B) Shows m/z = 736.9 spectrum at the 11.3 min retention time, based on the HPLC chromatogram presented in (A).
(C) Shows the MS/MS of m/z = 736.9 doubly charged ion together with the y and b ions assignments used to identify the Rho peptic peptide fragment F294AKTSAVYNPVIY.
(D) Provides a detailed view of the isotopic distribution of the undeuterated (0% D2O) m/z = 736.9 ion previously shown in (B) and identified in (C).
(E) Shows a detailed view of the isotopic distribution of fully deuterated (100% D2O) pepsin-digested Rho fragment F294AKTSAVYNPVIY.
(F) Hydrogen exchange plot of peptide fragment (F294AKTSAVYNPVIY) as a function of incubation time (s). The Rho hydrogen-deuterium exchange procedure and digestion parameters are described in the Experimental Procedures subsection “Amide Hydrogen-Deuterium Exchange of Rho.” Deuterium uptake is shown as a percentage calculated as described in the Experimental Procedures subsection “Analysis of Deuterium Exchange” (see also Table S1 and Figure 4). The percent uptake at different time intervals (0 to 1,800 s) for Rho is shown by filled black squares, for Rho* by filled red circles, and for the Rho*-Gt complex by filled blue triangles. Error bars represent SEM, and statistical significance was assessed by the Student’s t test. Data represent at least three independent experiments.
HPLC separation and MS analysis. This was achieved by lowering the sample and HPLC column temperature to ~2°C–4°C concomitantly with careful control of the sample pH. Hydrogen-deuterium exchange control experiments using synthetic peptides derived from the Rho primary sequence (C-terminal peptide and the chromophore binding site) spiked into explicitly measure back exchange determined that back exchange was minimal (~3%) during the pepsin digest period but was influenced by the retention time of the peptide on the column. This back exchange during the HPLC separation was found to be in the 10%–30% range (Figures 5A–5C). Typical plots (Figures 6A–6F; see also Tables S1 and S2, available online) were represented as color-coded uptakes in Rho and Gαs.

In essence, these back-exchange control experiments provide benchmarks for comparisons to protein experimental data.

For Rho purified in detergent-lipid mixed micelles, deuterium uptake was minimal. Normalized values for each state are shown in the supplement (Figures S1A and S1B). The only region with significant exchange was the C-terminal peptide T336VSKTETSQVAPA (Figure S1A, in red, compared to Figure S1B, in pink). The TM regions generally resisted exchange, with the exception of peptides in close vicinity to the chromophore binding site (F294AKTSAVYNPVIY), where exchange approached 30%. Following light activation of Rho, several regions had altered deuterium uptake profiles. Although the whole molecule showed increased deuterium uptake, several peptide fragments showed a considerable increase compared with the dark form of Rho (Figure 7A). These fragments were located in the cytoplasmic loops C-II and C-III (F146RFGENHAIMG and T229VKEAAAQQQESATTQ), the chromophore-binding site (F294AKTSAVYNPVIY), and the C-terminus (T336VSKTETSQVAPA).

Uptake in the TM regions of Rho* was in the range of 10% to 20% as opposed to <10% in the case of Rho. Comparing Rho in delipidated form with Rho with the sample containing phospholipids, we found that regions that show decreased uptake compared to Rho were located in the C—I and E—III domains (Figure 7B). Interestingly, more TM regions seem to have decreased uptake upon activation (Figure 7B, in green).

When lipids were completely removed during the purification procedure, the differences between Rho and Rho* were less prominent although exchange overall was consistently higher. For example, differences in the TM-I, TM-II, and C-I regions (F146RFGENHAIMG and T229VKEAAAQQQESATTQ), the chromophore-binding site (F294AKTSAVYNPVIY), and the C-terminus (T336VSKTETSQVAPA).

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When lipids were completely removed during the purification procedure, the differences between Rho and Rho* were less prominent although exchange overall was consistently higher. For example, differences in the TM-I, TM-II, and C-I regions were insignificant, but some peptides from TM-IV showed increased deuterium uptake in the Rho* form (Figure S1D, green; compare to Figure S1C, in dark cyan; compare to Figure S1C, dark cyan). Another difference was in the C-terminal segment of Rho, which showed ~70% deuterium uptake (Figure S1D, in orange) as compared to ~80% in the same region of Rho* (Figure S1D, pink). Because this purification method for Rho was comparable with the Rho*-Gα complex method, we were able to compare these states. The Rho*-Gα sample overall showed a decreased uptake in deuterium (~50%, Figure 8A, normalized uptake by peptidest obtained from Rho samples is shown in white, by peptides obtained from Rho* samples in light gray, and by peptides obtained from Rho*-Gα, in dark gray. Deuterium uptake by the synthetic peptides F294AKTSAVYNPVIY and T336VSKTETSQVAPA, respectively. Deuterium uptake by the synthetic peptide F294AKTSAVYNPVIY and T336VSKTETSQVAPA in the presence of DDM is shown in black.
values for each state are shown in Figure S2); however, this was reflected as both increases and decreases in exchange for specific peptides compared to Rho and Rho*. The first extracellular loop (E–I) had uptakes of <20% in the case of Rho and Rho*, whereas the uptake in the Rho*-Gt complex was increased to ~40% (Figures S1C and S1D, in blue; compare to Figure S2A, in green). The E-II peptide W175SRYIPEGMQ had an uptake of 10% in the Rho state, an increase to 40% in Rho*, and reached 50% in the Rho*-Gt complex (Figure S1C, in cyan; Figure S1D, in green and Figure S2, in yellow). The TM-VI, E-III, and TM-VII regions in Rho* (Figure S1D) exhibited a decreased uptake when compared with Rho (Figure S1C). These regions become even more resistant to deuterium exchange in the Rho*-Gt complex (Figure 8A).

Comparison of the domain arrangement in the Rho*-Gt three-dimensional model obtained from electron microscopy images with the available X-ray structure of the β2-AR showed a major disagreement in the organization of the α domain (Figures 8B...
and 8C). Hence, we focused our analysis to the α domain of Gt. When Gt was in complex with Rho*, the deuterium uptake of the Gt subunit was found to be increased as denoted by the positive differences between the Rho*-Gt state versus the free Gt state (Figures 9A and 9B). Normalized values for each state are shown in Figure S3. In contrast, deuterium uptake of free Gt was found to be reduced when compared to Gt in the (Rho*)2-Gt complex (Figures S3B and S3C). For the majority of the peptic peptides resulting from the pepsin digest of Gt, maximal uptake was obtained after only 30 s of D2O incubation as opposed to the 10 min incubation needed in the case of the Rho or Rho* molecule. The largest changes were observed for the following Gt fragments: A7EEKHSRELEKKLKEDEKDART (31%), L33LLGAGESGKSTIVK (38%), L33LLGAGESGKSTIVKMKIIHQDGYSLE (22%), E157RLVTPGYVPTEQD (59%), and T321CATDTQNVK (32%).

**DISCUSSION**

We first investigated different preparations of Rho. We found major differences in hydrogen-deuterium uptake among different states of Rho (Figure 7). These differences are attributed to the presence or absence of lipids or activation of Rho. We have also investigated the (Rho*)2-Gt complex reconstituted from native purified proteins and subjected it to hydroxyl radical footprinting and hydrogen-deuterium exchange. Our results directly identified reorganization of water molecules in different states of Rho* and identified dynamics regions in both Rho* and Gt. The observed rates of hydrogen-deuterium exchange were compared to control experiments, where individual proteins were investigated. Because the complex is highly delipidated, the Rho sample in either delipidated or in the mixed micelles of detergent and lipids were also compared with each other. Finally, we identified the conformational dynamics of activation for the pentameric complex of dimeric Rho* and heterotrimeric G protein.

Although Rho* in the Rho*-Gt complex exhibits the same UV-vis spectral properties as isolated Rho* (Jastrzebska et al., 2011c), the conformations of both states most likely are not the same. Indeed, Fourier transform infrared (FTIR) studies of the interaction between Rho* and Gt-derived peptide revealed that formation of the complex between these two entities is accompanied by structural changes in both (Vogel et al., 2007). This indicates that the conformation of Rho* in the Rho*-Gt complex differs from that of isolated Rho*.
Figure 8. Differences in Normalized Hydrogen-Deuterium Exchange between Rho*-Gt Complex and Rho* Purified in the Presence of Detergent

(A) Shows the differences in normalized deuterium uptake between Rho*-Gt and Rho purified in the presence of detergent. Calculation of the differences in normalized deuterium uptake and the color-coding employed were identical with what was described for Figure 7.

(B) Shows a recent model of the Rho* interaction with Gt constructed from electron microscopic images (Jastrzebska et al., 2011c). The two monomers of Rho* are shown in red and yellow (front view and a 90° rotated view). The palmitoyl chains are shown as pink sticks, whereas the Gt molecule domains are shown in blue, green, and cyan. Chains of the Gt protein are colored as follows: Gα in blue; Gβ in cyan; and Gγ in green.

(C) Shows an X-ray structure of the β2AR-Gs complex (PDB 3sn6; Rasmussen et al., 2011). The β2AR is shown in yellow, whereas the coloring of the Gs chains is kept similar to that shown for the Gt molecule in (B). The T4 lysozyme and the Nanobody-35 present in the X-ray structure were deleted to facilitate easy viewing.

See also Figure S2.
Figure 9. Differences in Hydrogen-Deuterium Exchange of Free Gt and in Complex with Rho*.
(A) Illustrates a recently described Rho*-Gt model (Jastrzebska et al., 2011c). The Rho* dimer is shown in gray. The location of Gt in the complex is shown together with color-coding, indicating differences in percentage uptake (see also Table S2) between the Rho*-Gt and free Gt states as described in Figure 7. The Gt and Gt domains are shown in gray.
(B) Shows a 90° rotated view of the Gt, domain of Gt. See also Figure S3.

complex differs from its conformation in free Rho*. Although structures of Rho intermediates available to date provide snapshots of specific, static states of interest, the protein dynamics during the activation event are also of interest in understanding the activation mechanism. Derived from recent studies with solid state ²H NMR, a multistage activation mechanism is proposed in which retinal isomerization initiates fluctuation of the helices and cytoplasmic loops in the development of Rho* (Struts et al., 2011). This in turn will result in lowered efficiency in protein modification. M²⁸⁶, M²⁵³, and M²⁸⁸ are located within the TM-II, TM-VII, and TM-VI regions of Rho, respectively. The side chains of these residues are facing inwards and are located in the vicinity of the internal water molecular network of Rho. The importance of this internal molecular network is emphasized by the M²⁵⁷Y mutation, which results in a constitutively active Rho (Deupi et al., 2012). The recent X-ray structure of the constitutively active Rho molecule bearing the M²⁵⁷Y mutation argues that the internal-water-molecule-mediated bonding network is disrupted after the mutation and hence prevents the chromophore-induced helix movement (Deupi et al., 2012). Superposition of the dark-state Rho (Okada et al., 2004) with Rho* (Choe et al., 2011) shows a different organization of the internal water molecule network. Furthermore, amino acid residues M²⁸⁶, M²⁵³, and M²⁸⁸ are localized in regions that show conformational changes upon Rho activation. The activation process that involves conformational changes in TM-V and TM-VI could explain differences in the oxidation pattern of these residues. Although TM-II does not show conformational changes to the extent seen in TM-V and TM-VI, a careful analysis shows an increase in the root-mean-square displacement between the two states, especially in the kink of the helix located in the vicinity of M²⁸⁶.

Because hydrogen-deuterium exchange is favored in regions that are solvent exposed and have unstable or unstructured secondary structure, we expected that peptides exhibiting both characteristics within Rho would show deuterium uptake, whereas TM regions would be relatively protected. When Rho in a delipidated form was compared with Rho in mixed micelles, an increased deuterium exchanged was observed for peptides encompassing the C-I (Y⁶⁰VTVQHKLRTPLNYIL) and E-III (Y²⁷¹IFTHQGSDFGPFF) domains. When Rho was compared with Rho*, each purified in the presence of lipids, we observed a considerably higher deuterium uptake by Rho* (Figure 7A).

Specifically, higher deuterium exchange was observed in the case of Rho* in the C-terminus region, the cytoplasmic loops C-I, C-II, and C-III and extracellular loop E-III. The TM region showed similar increase in the activated form of Rho, except for a small region located on helix VI (in green). The N-terminus and the extracellular domain II were found to have less exchange in the Rho* state when compared to Rho.
This result suggests that lipids-associated Rho* is either highly hydrated (Jastrzebska et al., 2011b) or more unstructured, consistent with ²H NMR experiments (Grossfield et al., 2008). This difference between the Rho and Rho* was also significant for delipidated affinity purified proteins (Figure 7B). However in this case, the differences cannot be simply interpreted as in the first case. The reason is that in the delipidated samples several regions undergo increased deuterium uptake in the Rho*, but other regions have decreased uptake when compared to ground state Rho. Delipidation resulted in increased uptake in some regions and decreased uptake in others. This could be possibly attributed to the fact that the delipidated Rho samples would be preferentially in monomeric form, whereas the lipidated samples Rho would be mostly found in its oligomeric form (Borochov-Neori et al., 1983; Jastrzebska et al., 2006). These results also point out difficulties in interpretation of experimental data for samples that necessitate the use of detergent, like 3D structural modeling.

Our recent electron microscopic model of the Rho*-Gt complex (Figure 8B) differs from the X-ray structure of the β₂AR-Gt protein complex (Figure 8C) in two important ways. First, with respect to the Rho*-Gt complex, the envelope obtained from electron microscopic reconstruction closely accommodates one Rho dimer molecule together with one β₂Gt protein heterotrimer. Second, the orientation of the Gt protein is different in the Rho*-Gt complex than in the β₂AR-Gt complex (Figures 8B and 8C). Although the resolution of the X-ray structure of the β₂AR-Gt protein complex is the highest to date (3.2 Å for a GPCR in complex with its Gt protein; Rasmussen et al., 2011) and offers a molecular view of its mechanism of action, the crystallization required the presence of the β₂AR N-terminally fused T4 lysozyme and Nanobody-35. The specific influence that these additions have on the final crystallographic packing is not known. The other working model of a GPCR with its Gt protein, based on electron microscopy and has a resolution of 21.6 Å. This was obtained without any other protein adjuvant, thereby considerably lowering the chance of artifacts introduced into the final structure (Jastrzebska et al., 2011c). Differences are also observed in the opening between the two Gt protein domains, which consists of a ~160° rotation of the α-helical domain from the Ras-like domain within the α subunit (Jastrzebska et al., 2011c; Rasmussen et al., 2011). However, the nucleotide-free complex would be short-lived to accommodate such a large conformation opening and the rebound nucleotide (GTP) will cause rapid complex dissociation to individual proteins.

For example, Majumdar et al. (2004) found that the exchange of GDP-GTP for the Rho*-Gt complex was 13.2 s⁻¹ under physiological conditions.

Thus, the C-terminal region in Rho with the sequence T³⁵⁶VSKTESQVAPA (Figure 2A) is an unstructured sequence expected to be solvent exposed (Cai et al., 1997; Langen et al., 1999; Palczewski et al., 1991); indeed, this peptide fragment exchanged 80% of its theoretically maximum exchangeable sites in both Rho and Rho* (Figures S1A–S1D). However, deuterium uptake by the same peptide sequence was reduced in the Rho*-Gt complex, revealing decreases in solvent accessibility or increases in the ordering of this region or both (Figure 8A). Other studies have suggested that the region close to the T³⁵⁶VSKTESQVAPA peptide is involved in the interaction with the Gt protein (Phillips and Cerione, 1994). This positioning of peptide T³⁵⁶VSKTESQVAPA in the Gt interaction site may account for the decreased exchange pattern in the Rho*-Gt complex.

As for the TM domains, most peptide fragment sequences we examined did not exchange deuterium as expected, for example, peptide M²⁸⁸TIPAF, but there also were exceptions, such as F³⁵⁹AKTAVNPV and M³⁸⁶TIPAF, transmembrane regions (FM³⁴⁶VFQGF), had considerably higher modification rates when compared with their counterparts in Rho or Rho*.

In lipid-free purified Rho, the regions from C-III to TM-VII become more resistant to deuterium exchange in the Rho* state. This reduced uptake could be attributed to formation of a secondary structural element as indicated in the X-ray structure of the Rho* state (Jastrzebska et al., 2011b). A recent study from our laboratory with ¹⁸O-labeled water found that water from bulk solvent, but not tightly bound internal water, is involved in the release of chromophore from its Rho binding site following activation (Jastrzebska et al., 2011b). Access of bulk water to the TM region of Rho could explain the observed deuterium uptake in these transmembrane regions. Deuterium uptake is also reduced when a peptide sequence adopts a secondary structure, such as is the case for a TM domain that exists as an α-helix. In this context, our results provide evidence that the region encompassing amino acids F³⁵⁹ to Y³⁰⁶ behave similarly to a longitudinal stretch along the axis of the α-helix in the seventh TM.

In conclusion, complementary radiolabeled footprinting and deuterium exchange studies of purified bovine Rho, Rho*, and Rho*-Gt complexes suggest that Gt protein binding imposes a different structural state of Rho as compared to its activated or ground states. We have also demonstrated that changes in detergent versus mixed micelles of detergent and phospholipids influence the conformation of Rho, suggesting the importance of investigating the complex in a native membrane environment. The apparent differences in hydroxyl radical labeling and deuterium uptake between Rho* and the Rho*-Gt complex suggest that light exposure causes structural relaxation of Rho followed by tightening upon Gt coupling. Allosteric communication between the chromophore binding site in Rho* and nucleotide-binding site of the Gt protein at a distance of ~50–60 Å is achieved by subtle conformational changes that include also reorganization of internal water molecules as demonstrated in this study. Our results also suggest that binding of Gt confers more stabilizing effect on Rho* that resembles Rho in its rigidity. This observation stands in sharp contrast to the highly dynamic structure of Rho* that forms a constellation of conformers.
**Structural Dynamics of Rhodopsin-Transducin Complex**

**EXPERIMENTAL PROCEDURES**

**Purification of Rho, Gt, and Rho-Gt Complex**

Bovine ROS membranes were prepared from fresh retinas under dim red light by step sucrose gradients as described in Papastergiou (1982). Rho was purified from ROS membranes solubilized in DDM by a ZnCl2-opsin extraction procedure (Ökada et al., 2000). ZnCl2 was then removed by dialysis in the presence of 0.2 mM DDM. Gt was purified from dark-adapted bovine ROS membranes by extraction with hypotonic buffer as described elsewhere (Goc et al., 2008). The Rho-Gt complex was purified by succinylation-Concanavalin A affinity chromatography as described in Jastrzebska et al. (2011).

**Radiolitic Footprinting**

A similar workflow sequence for footprinting experiments was used for Rho, Rho*, and the Rho-Gt complex purified by scCoA resin. Experiments were initiated by exposure of each sample to high-flux synchrotron X-ray radiation at the beamline X28C of National Synchrotron Light Source as in our previous studies (Angel et al., 2009; Orban et al., 2010). Exposure times were set to 0, 1, 2, 5, and 5 ms and were controlled by a Kinet apparatus described elsewhere in detail (Gupta et al., 2007, 2010). Samples then were quenched by addition of methionine amide (100 mM, final concentration) and each 100 μl aliquot was flash frozen with dry ice and stored at −80 °C. Samples (100 μl) were chemically reduced by the addition of DTT (5 μM, final concentration) for 45 min at 37 °C and alkylated for 1 hr in the dark with iodoacetamide (10 μM, final concentration) dissolved in 25 mM NH4HCO3. Proteins were precipitated by addition of 400 μl acetone and kept at −80 °C overnight. Protein pellets were resuspended in 5 μl of formic acid, and the volume was raised to 100 μl with water. Next, samples were digested with pepsin overnight at 23 °C. Samples were desalted with a C18 UltraMicro Tip column (The Nest Group Inc., Southborough, MA, USA). Liquid chromatography-tandem MS (LC-MS/MS) was performed with a LTO-Orbitrap XL linear ion trap MS (Thermo Finnigan, Bremen, Germany) equipped with a nanospray source operated in a positive ion mode. HPLC separation was accomplished with an UltiMate 3000 parallel LC system (Dionex Inc., Sunnyvale, CA, USA). The protein digest (5 μl) was injected onto a reversed-phase C18 PepMap trapping column (0.3 mm × 5 mm, 5 μm particle size; Dionex Inc.) and washed with 0.1% formic acid. Peptides were then eluted off the trap column onto a reverse-phase C18 Acclaim PepMap 100 column of 0.075 × 150 mm (Dionex Inc.) by using a gradient of acetonitrile running from 5% to 60% in 0.1% formic acid over a period of 60 min at a flow rate of 300 nL/min. Spectra were acquired in a data-dependent manner consisting of a full scan followed by a MS/MS scan of the five most abundant precursor ions at the normalized collision energy of 30%. Dynamic exclusion was applied to exclude multiple MS/MS analyses for the same peptide. Identification of peptides was achieved with the online version of MassMatrix, and the data were searched with the Rho primary sequence used as the database. Parameters chosen for peptide identification were error tolerance for precursor ion m/z values set at 10 ppm, error tolerance for the fragmented product ion m/z values set at 0.8 Da, mass type set to monoisotopic, and maximum peptide length set at 40 amino acids. The quality of the peptide search for modified and unmodified peptides was evaluated by three statistical scores: pp, pp22, and ppwq set at 5, 5, and 1.3, respectively, as described in detail elsewhere (Xu and Chance, 2007). Peptide fragments of Rho were identified by MS/MS analysis. The score for each peptide was evaluated and checked for statistical significance. All reported peptides were identified with a p value < 0.005. Side-chain modifications were introduced within the MassMatrix search criteria based on values previously reported (Xu and Chance, 2004, 2005; Xu et al., 2003). Following identification of the modified and unmodified peptide fragments, the fraction unmodified was calculated for each exposure time by using the following formula:

\[
\text{Fraction unmodified} = \frac{A_u}{A_u + A_m}
\]

where \(A_u\) represents the area under the total ion current of the unmodified peptic peptide fragment, and \(A_m\) represents the area under the total ion current of the modified peptic peptide fragment. The area under the total ion current was evaluated based on a semiautomatic procedure implemented in Proteome Discoverer (Thermo Fisher Scientific). A semiautomatic procedure implemented in ProDiscoverer (Thermo Fisher Scientific) was used to calculate the fraction of unmodified peptides at each exposure time. The results were then compared with those obtained from a manual analysis of the raw data. Statistical significance of the identified peptides was evaluated based on scoring described elsewhere (Xu et al., 2008). Peptides reproducibly identified are listed in Table S1; these were used to construct the progress plots. Raw data in the form of relative signal intensity (%) as a function of m/z were extracted using XCalibur 2.1.0. Qual Browser was used for a semiautomatic peak detection and deconvolution procedure performed with HDExpress software (Weis et al., 2006) just recently described (Orban et al., 2010a). Briefly, after deuterium uptake was evaluated from the raw data, the value for each peptide fragment was normalized to 80% of the theoretically maximum exchangeable sites to account for the 80% deuterium accomplished by the experimental setup (see Table S1, column 3). For calculations of the maximum exchangeable sites, only peptide bonds were used to account for the amide exchange; deuterium exchange from side chains was considered negligible and was not included. The number of maximum exchangeable sites was decreased by one for each P residue in a peptide sequence. Hydrogen-deuterium exchange was color-coded based on the total percent of the theoretical.

**Amide Hydrogen-Deuterium Exchange of Rho**

These experiments were done in the dark under dim red light. Amide hydrogen-deuterium exchange was performed as follows. About 25 μg of ZnCl2-opsin-extracted Rho, affinity-purified Rho, Rho samples exposed to light for 30 s through a 480–520 nm band pass filter (150 Watt filter light; Dolan Jenner Industries Inc., Boxborough, MA, USA) or purified Rho-Gt complex were diluted in 80 μl D2O containing 0.2 mM DDM and kept on ice for 10, 30, 60, 300, 600, or 1,800 s. After exposure to deuterated buffer, the exchange was terminated with ice-cold quenching buffer composed of D2O and 0.2 mM DDM (pH 2.5). Ten microliters of peptide (1.7 mg/ml; Worthington, Lakewood, NJ, USA) was immediately added to the solution, and the sample was digested for 10 min on ice. Next, the sample (100 μl) was loaded on a Luna 20 × 2.0 mm C18 column (Phenomenex, Torrance, CA, USA) with a temperature-controlled Hewlett-Packard autosampler set to 4 °C. All tubes containing samples (Rho, Rho*, and Rho-Gt) were then washed in aluminum foil, remained unexposed to ambient light, and the HPLC column was kept submerged in ice during the experiment. Peptides were eluted with the following gradient sequence: 98% H2O with 0.1% (v/v) formic acid (A) and 2% acetonitrile with 0.1% (v/v) formic acid (B); 4–12 min, 98% to 2% A. Separation was performed with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA), and the flow rate was 0.2 mL/min. The eluent was injected into a Thermo Finnigan LTQ (Thermo Scientific, Waltham, MA, USA) MS equipped with an electrospray ionization source operated in the positive ion mode with other parameters adjusted as follows. Activation type was set to collision-induced dissociation, normalized collision energy to 35 kV, capillary temperature to 370 °C, source voltage to 5 kV, capillary voltage to 43 V, the tube lens to 105 V, and MS spectra were collected over a 200–2,000 mass range. To avoid sample propagation from one HPLC run to another, each production run was followed by a mock injection of 10 μl of solution A with a resulting HPLC elution profile identical to the one described previously. This run was followed by a 20 min equilibration run with 98% A and 2% B for 20 min. Experiments were performed at least in triplicate for each time point. Experiments for free Gt were conducted under identical conditions as described previously.

**Analysis of Hydrogen-Deuterium Exchange**

Peptides resulting from the peptic digestion were identified by MS/MS employing the online version of MassMatrix (Xu and Freitas, 2008b). Search criteria used to identify a peptic digest were as follows. Precursor ion tolerance was set to 0.8 Da, the maximum number of variable modifications allowed for each peptide sequence was set to 2, the minimum peptide length was set to 4 amino acids, the minimum pp score was set to 5, the ppwq score was set to 1.3, the maximum number of combinations of different modification sites for a peptide match with modifications was set to 1, and the maximum number of candidate peptide matches for each spectrum output in the result was set to 1 (Xu and Freitas, 2007). The product ion tolerance was set to 0.8 Da, and the mass type was set to average. These search criteria were extended to include Rh glycosylation sites at N2 and N15, the disulfide bond between residues C115 and C187, and the palmitoylation sites at C229 and C233 (Table S1, modified residues are shown in bold and underlined). Statistical significance of the identified peptides was evaluated based on scoring described elsewhere (Xu et al., 2008). Peptides reproducibly identified are listed in Table S1; these were used to construct the progress plots. Raw data in the form of relative signal intensity (%) as a function of m/z were extracted using XCalibur 2.1.0. Qual Browser was used for a semiautomatic peak detection and deconvolution procedure performed with HDExpress software (Weis et al., 2006) just recently described (Orban et al., 2010a). Briefly, after deuterium uptake was evaluated from the raw data, the value for each peptide fragment was normalized to 80% of the theoretically maximum exchangeable sites to account for the 80% deuterium accomplished by the experimental setup (see Table S1, column 3). For calculations of the maximum exchangeable sites, only peptide bonds were used to account for the amide exchange; deuterium exchange from side chains was considered negligible and was not included. The number of maximum exchangeable sites was decreased by one for each P residue in a peptide sequence. Hydrogen-deuterium exchange was color-coded based on the total percent of the theoretical.
maximum deuterium uptake at 10 min as follows: dark blue, 0%–9%; blue, 10%–19%; light blue, 20%–29%; cyan, 30%–39%; green, 40%–49%; yellow, 50%–59%; orange, 60%–69%; red, 70%–79%; pink, 80%–100%. The same procedure was employed for the free Gq samples as described previously.

Evaluation of Back Exchange

Three synthetic peptides were designed to evaluate the back exchange of authentic peptide standards. These peptide sequences mimicked fragments from Rho’s primary C-terminal structure (T106VSKTETSTQVAPA), chromophore binding site (F(339)AKTSAYNPVYI), and transmembrane region (M(288)TIPAF). Procedures for sample preparation, HPLC elution conditions, MS parameters, and MS/MS identification of the peptides with MassMatrix (Xu and Freitas, 2009b) were identical to those described under the Experimental Procedures subsection “Amide Hydrogen-Deuterium Exchange of Rho.”

Statistical Analyses

Graphical and statistical analyses were carried out with Origin 8 SR0 software (version 8.0725, OriginLab Corporation, Northampton, MA, USA). Error bars represent standard errors of the means (SEM) and statistical significance between photoactivated rhodopsin and transducin: progress and questions. FASEB J. 2012 Elsevier Ltd All rights reserved.
Structure

Dynamics of Rhodopsin-Transducin Complex


