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Monomeric G-Protein-Coupled Receptor as a Functional Unit

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ABSTRACT: Rhodopsin, the first purified G-protein-coupled receptor (GPCR), was characterized as a functional monomer 30 year ago, but dimerization of GPCRs recently became the new paradigm of signal transduction. It has even been claimed, on the basis of recent biophysical and biochemical studies, that this new concept could be extended to higher-order oligomerization. Here this view is challenged. The new studies of rhodopsin and other simple (class 1a) GPCRs solubilized in detergent are re-assessed and are compared to the earlier classical studies of rhodopsin and other membrane proteins solubilized in detergent. The new studies are found to strengthen rather than invalidate the conclusions of the early ones and to support a monomeric model for rhodopsin and other class 1a GPCRs. A molecular model is proposed for the functional coupling of a rhodopsin monomeric unit with a G-protein heterotrimer. This model should be valid even for GPCRs that exist as structural dimers.

The G-protein-coupled receptors (GPCRs)[1] constitute the largest known family of cell membrane receptors. They sense a large variety of sensory, chemotactic, hormonal, and neuronal signals, which they transduce across the cell membrane by coupling to heterotrimeric G-proteins. Their basal structure is a seven-transmembrane α-helix (7TM) domain. Historically, rhodopsin was the first 7TM receptor to be purified to homogeneity, ∼30 years ago, and its functional coupling to transducin, the first isolated heterotrimeric G-protein ($G_t\alpha\beta\gamma$), was demonstrated in the early 1980s (*1*, *2*). Thousands of GPCRs have now been isolated and characterized. They all share the same basal architecture, which consists of a seven-transmembrane helix core domain, caped on the external side by a ligand binding domain and on the cytoplasmic side by a domain that interacts with heterotrimeric G-proteins and other intracellular effectors.

All GPCRs were initially assumed to be monomeric and to couple to one G-protein heterotrimer. This coupling is transient: it catalyzes the exchange of GDP for GTP in the Gtα subunit of the bound G-protein, which then dissociates from the receptor in separate active $G_t\alpha_{GTP}$ and $G\beta\gamma$ subunits that diffuse away to contact their various effectors. Thus, a single activated receptor can activate sequentially a large number of G-proteins. Variations were, however, soon observed among the GPCR structures. Around the conserved motif of the seven-transmembrane helix core domain, long extensions in the N-terminus may fold in large extracellular domains. Extensions are also seen in the cytoplasmic C-terminus and long insertions in the third cytoplasmic loop. When these variations are taken into account, the GPCRs

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[1] Abbreviations: cmc, critical micellar concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; $C_{12}E_8$, dodecanoyl octaoxyethylene glycol; DDAO, dodecyldimethylamine oxide; DDM, dodecyl maltoside; GABA, γ-aminobutyric acid; GPCR, G-protein-coupled receptor; $Gt\alpha\beta\gamma$, photoreceptor heterotrimeric G-protein transducin; R*, photoactivated rhodopsin; 7TM, seven-transmembrane α helices.

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can be sorted into several subclasses (*3*). The simplest, with short N- and C-termini, form class 1a receptors for small ligands that insert within the 7TM domain; this includes rhodopsin and numerous odorant receptors. Class 1a GPCRs were generally considered as functional monomers, but some of them are able to form homodimers, or heterodimers with another GPCR of the same class, via their C-termini, or via contacts between the two 7TM cores. The most complex GPCRs form class 3, which includes mainly the glutamate metabotropic receptors and the GABA receptors. They have a long N-terminal extension folded in separate extracellular domains, and a C-terminal extension. They are constitutive homo- or heterodimers, linked by S−S bridges and associated mainly by their large extracellular domains, which include the ligand-binding site.

In all cases, dimerization seems not to be required for functional coupling of the GPCR to heterotrimeric G-protein, but mainly for its intracellular trafficking: addressing the cell membrane of newly synthesized receptors and processing of the activated receptors by arrestin and adaptor proteins prior to endocytosis (*4*, *5*). Heterodimerization may also affect the pharmacological specificities of receptors (*6*). But even for class 3 receptors, dimerization of the 7TM domain does not seem to be critical for the nominal function of the activated GPCR which is to couple to the G-protein. In the best documented class 3 GABA receptor, both extracellular domains of the constitutive heterodimer are involved in the process of receptor activation, upon binding of an agonist to one of them, but only one of the two 7TM domains plays a role in the coupling of the dimeric GPCR to its cognate G-protein (*7*, *8*).

Despite these known limitations on its generality and on its functional significance, dimerization of GPCR has recently become the new paradigm of signal transduction by all 7TM receptors and heterotrimeric G-proteins. This concept is presented more and more assertively in successive reviews and is often extended to higher-order oligomerization. In the recent review in *Biochemistry* (*9*) entitled Oligomerization of G Protein-Coupled Receptors: Past, Present, and Future, Park, Filipek, Wells, and Palczewski state that, "recent biochemical and biophysical studies have challenged the traditional concept of monomeric GPCRs and point instead to a mechanistic view of signal transduction wherein the receptor functions as an oligomer." They note that, "reports on the oligomerisation of GPCRs continue to accumulate at a rapid pace" and conclude that "this prevalence of oligomers calls into question the role and even the existence of monomers in nature". But we remind the reader that reports on rhodopsin, the first GPCR ever purified, had also accumulated in the 1970s and 1980s. All these reports had rigorously characterized rhodopsin as a monomeric protein on the basis of a variety of biophysical and biochemical studies. This had already led to a very mechanistic view of signal transduction.

The recent biophysical studies quoted above referred essentially to the work of the groups of Engel and of Palczewski (*10*) and subsequent papers (*11*−*13*), which provided spectacular images of rhodopsin quasi-crystalline arrays observed by atomic force microscopy on retinal rod disk membranes in vitro. However, it was not clear that this represented the native arrangement of rhodopsin in vivo, and the significance of these atomic force microscopy studies has been questioned by some of those which had contributed to the early biophysical studies on retinal rods (*14*).

In this review, we re-assess the "recent biochemical studies" quoted by Park et al. (*9*) as evidence of the oligomerization of rhodopsin-like GPCRs. These refer to new hydrodynamic and functional characterizations of rhodopsin (*15*, *16*) and other class 1a GPCRs in detergent (*17*, *18*), performed over the past two years. But extensive hydrodynamic and structural characterization of detergent-solubilized rhodopsin had also been performed in the 1970s and 1980s, and in all hands, including ours, these studies had consistently resulted in the characterization of rhodopsin as monomeric, at least in its dark, unactivated state. Thus, before assessing the new biochemical studies, we first must review these "old" biochemical and functional characterizations of rhodopsin in detergent micelles.

Early Studies of Rhodopsin in Detergent

Characterization of solubilized membrane proteins started with the pioneering works of Helenius and Simons (*19*) and Tanford and colleagues (*20*−*22*). Visual rhodopsin was one of the first intrinsic membrane proteins to be purified to homogeneity in detergent. The choice of detergent was based on the solubilization efficiency of rod outer segment membranes and on the fact that the native absorption spectrum was maintained. The complex that rhodopsin forms with Triton X-100 was first characterized hydrodynamically by Osborne, Sardet, and Helenius (*23*). In that detergent, the rhodopsin−detergent complex was found to consist of one protein monomer and a large number (80) of detergent molecules. The monomeric rhodopsin model was by no means a traditional concept. At the time, visual rhodopsin was thought to be structurally and functionally related to bacteriorhodopsin, which is organized as a trimer in the *Halobacterium* membrane.

Further extensive hydrodynamic and structural characterization of the complex of rhodopsin with detergent was performed by gel filtration chromatography, sedimentation velocity analysis, and small-angle neutron scattering (*24*). The study extended to other nonionic detergents such as dodecyldimethylamine oxide (DDAO) and a C12/C14 detergent with a polyoxyethylene glycol polar head (Cemulsol LA90). Partial deuteration of the detergent and adjustment of the D_2O:H_2O ratio in the solvent eliminated the detergent contribution to the scattering and permitted direct measurement of the protein molecular mass, which was found to correspond exactly to that of a single rhodopsin monomer. Further measurements at other solvent contrasts indicated that ∼110 molecules of Cemulsol LA90 detergent were bound to rhodopsin. As with the hydrodynamic characterizations, this value was independent of the shape of the complex.

The small-angle neutron scattering studies had been carried out on dark-adapted, i.e., nonactivated rhodopsin. The functionality of the rhodopsin monomers in detergent could not be assayed at the time, as the GPCR function of rhodopsin had not yet been elucidated. An important observation, however, was that flash photoactivation of rhodopsin monomers in a detergent with a polyoxyethylene glycol polar headgroup induced on a time scale of minutes a doubling of the scattering intensity that is characteristic of protein dimerization (*24*). The slow kinetics of this apparent

dimerization excluded the possibility that the rhodopsin dimer could have a role in visual signal transduction, which occurs on a subsecond time scale. This slow dimerization was followed by an even slower oligomerization that correlated with flocculation and precipitation. The protein had denatured upon photoactivation in this detergent. The extent and kinetics of oligomerization were later shown to depend on the type of detergent. Denaturation was minimal in detergents with linear alkyl chains and sugar polar headgroups, such as sucrose monolauryl ester or dodecyl maltoside. Importantly, dimerization and oligomerization of rhodopsin upon illumination had never been observed in the native membrane in the absence of detergent, using the same technique of small-angle neutron diffraction (*25*).

In 1981, McCaslin and Tanford (*26*) demonstrated by analytical ultracentrifugation a homogeneous population of monomeric rhodopsin in dodecanoyl octaoxyethylene glycol ($C_{12}E_8$), while in cholate, rhodopsin formed oligomers of various sizes. The smallest oligomeric species were trimeric, but higher-order oligomers, at least up to hexamers, were also present. In both detergents, the native absorption spectrum of the unbleached protein was maintained; however, upon photolysis, the $C_{12}E_8$ preparation aggregated and lost its ability to recombine with 11-*cis*-retinal, whereas the preparation in cholate did not. When bound $C_{12}E_8$ was replaced with cholate, the monomeric preparation became oligomeric.

The interaction of detergents with membrane proteins has been assessed by both biochemical and hydrodynamic methods (*27*, *28*). On the basis of frictional ratio measurements, the monomeric model for rhodopsin in Triton X-100 and $C_{12}E_8$ was confirmed (*27*). For other large membrane proteins, the number of bound detergent molecules is not the same as the detergent micelle aggregation number; it is usually larger (*28*).[2]

The late biochemist Hermann Kühn was the first to demonstrate, by simple binding experiments, a functional interaction of a 7TM receptor, photoactivated rhodopsin (R*), with a heterotrimeric G-protein, transducin (Gt$\alpha\beta\gamma$) (*1*). He also determined the kinetics and stoichiometry of this interaction in the native membrane using near-infrared light scattering (*29*). Upon rhodopsin photoactivation, the G-protein interacts with R*, forming a stoichiometric 1:1 complex, R*−Gt$\alpha\beta\gamma$, which is stable in the absence of GTP. In the presence of GTP, the complex rapidly dissociates, liberating the GtαGTP and Gt$\beta\gamma$ complexes. The resulting free R* then rapidly reassociates with another Gt$\alpha\beta\gamma$ heterotrimer, and the process is repeated. Thus, one R* monomer can sequentially activate hundreds of Gt$\alpha\beta\gamma$ molecules, at a rate close to one per millisecond (*30*). Kühn further biochemically analyzed the interaction between purified Gt$\alpha\beta\gamma$ and purified rhodopsin in a detergent solution. The detergent chosen was sucrose monolauryl ester, in which both dark-adapted and photoactivated rhodopsin are monomeric. Dark-adapted rhodopsin−detergent complexes were bound to a concavallin A−Sepharose column, and purified holotransducin Gt$\alpha\beta\gamma$ was applied to the column. Transducin did not bind to columns that had been loaded with inactive rhodopsin and kept in the dark, but did bind to columns loaded with photoactivated rhodopsin. Bound Gt$\alpha\beta\gamma$ was subsequently eluted upon addition of GTP or GTPγS, which fully demonstrated the functionality of detergent-solubilized, monomeric rhodopsin. This experiment was reported in some detail in a review on light-dependent interaction of rhodopsin with proteins in rods (*31*). However, the specialized book in which it was published is difficult to obtain. In this review, Kühn mentions that further quantitative measurements of the interaction of detergent-solubilized, monomeric rhodopsin with transducin had been performed, which he intended to publish *in extenso*. Publication did not seem to be urgent at the time as the monomeric model for rhodopsin was already well accepted. Instead, Kühn became more interested in the interaction with rhodopsin of other proteins, such as rhodopsin kinase and arrestin, and a few years later, this great pioneer of rhodopsin biochemistry accidentally died.

The stoichiometric complex between photoexcited rhodopsin and transducin was further characterized by Bornancin et al. (*32*), who demonstrated the extreme stability of the R*−Gtα(empty)Gt$\beta\gamma$ complex when the medium is depleted of guanine nucleotide. The monomeric character of rhodopsin in such a complex was not a disputed issue at the time of this study.

Thus, in the early 1980s, the monomeric status of rhodopsin in detergent seemed to be universally accepted. However, in 1981, the review article in *Nature* entitled Membrane Protein Oligomeric Structure and Transport Function by Klingenberg (*33*) opened on the strong statement that, "Proteins which traverse membranes tend to have a dimeric structure", which may have initiated the current craze regarding GPCR dimerization. But it should be noted that in this very article rhodopsin is cited as the counter-example of a membrane protein that occurs as a monomer either in the membrane or in the isolated, detergent-solubilized state.

Recent Studies of Rhodopsin and Other Class 1a GPCRs in Detergent

Let us now review the four recent biochemical studies in detergent that are quoted by Park et al. (*9*) as evidence of the oligomerization of rhodopsin-like GPCRs.

(1) Medina et al. (*15*) investigated the oligomeric state of rhodopsin using cross-linking techniques both in retinal rod outer segment membranes and after purification in dodecyl maltoside (DDM). Dimers, trimers, and higher-order oligomers were detected by SDS−PAGE, after cross-linking rhodopsin within the retinal membranes for 30−60 min. This was an expected result due to the high rhodopsin concentration in the retinal membrane. However, the monomer remained the major species; therefore, the result was inconclusive. A single oligomeric species was not present, and monomeric rhodopsin may have been nonspecifically cross-linked. It would have been more informative to check whether high DDM concentrations would prevent cross-

[2] Measurements performed on several membrane proteins and detergents (*28*) indicated that detergent binding can be a measure of hydrophobic surface area of integral membrane proteins. On the basis of that idea, it is likely that, on that account also, rhodopsin is monomeric in Triton X-100 and $C_{12}E_8$. Indeed, the amount of Triton X-100 and $C_{12}E_8$ bound to rhodopsin is similar to that observed for monomeric bacteriorhodopsin or Ca^{2+}-ATPase in these detergents (*27*, *28*). Since both proteins have a number of transmembrane segments identical or similar to rhodopsin (7 and 10, respectively), it would be very unlikely that rhodopsin would be dimeric and the others monomeric.

linking, which would have strongly indicated the presence of monomers [this approach was later used by Jastrzebska et al. (*16*)]. Interestingly, they also showed that light-activated, purified rhodopsin was partially dimeric even in the absence of cross-linking agents (refer to their Figure 1B).

A second experimental approach also suggested that DDM-solubilized rhodopsin was dimeric. Calibrated gel permeation column chromatography, measurement of DDM binding, and sedimentation velocity each seemed to indicate that DDM-solubilized rhodopsin was dimeric. Unfortunately, their study was flawed by a number of conceptual mistakes, and their conclusions were not valid. First of all, the gel filtration column was calibrated in terms of molecular mass, using water-soluble proteins, a procedure that is known to be unreliable with membrane proteins (*20*, *34*). The mass of a pure DDM micelle (50 kDa) was subtracted from the apparent mass (126 kDa) to obtain the mass of the protein (76 kDa), which corresponds approximately to dimeric rhodopsin. As previously demonstrated (*28*, *35*), this procedure cannot be used to determine the mass of membrane proteins of this size,[3] since the mass of the bound detergent is not necessarily equivalent to the micellar mass, but is often quite different. In fact, using the detergent binding value of Medina et al. (0.97 g of DDM/g of protein),[4] a rhodopsin dimer should bind ~80 kDa of DDM, and the above subtraction method would result in a very different conclusion (approximately a monomer). Note also that the Stokes radii reported by Medina et al. for unbleached and light-activated rhodopsin in DDM (4.18 and 4.15 nm, respectively) are somewhat smaller than the value for monomeric rhodopsin in either Triton X-100 or $C_{12}E_8$ [4.8 nm (*34*)]. It is our experience that membrane proteins have similar Stokes radii in $C_{12}E_8$ and in DDM [e.g., 5.5 nm for the sarcoplasmic reticulum Ca^{2+}-ATPase (*36*)], and it is unlikely that the smaller Stokes radius reported by Medina et al. corresponds to dimeric rather than monomeric rhodopsin.

Several other inconsistencies within the Medina et al. study (*15*) are mentioned. (i) The authors erroneously use *isopycnic* sedimentation to measure sedimentation coefficients. As pointed out long ago by Tanford et al. (*20*), sucrose gradient centrifugation should be used with great caution in measuring the sedimentation coefficients of detergent-solubilized membrane proteins. (ii) Their calibration of M versus the sedimentation coefficient times the Stokes radius (mentioned in the text, but not shown) is meaningless; it ignores the buoyancy factor. (iii) The cited f/f_o value cannot be calculated from their data. (iv) The hydrodynamic reasoning is valid whatever the shape of the complex, and it is not necessary to assume a globular and compact shape for the protein−detergent complexes, as they state. (v) Finally, and very surprisingly, none of the previous articles on solubilized rhodopsin were cited. In almost every case, these articles had concluded that rhodopsin was monomeric.

(2) Jastrzebska et al. (*16*) also used cross-linking agents to determine the oligomeric state of rhodopsin within native retinal rod outer segment membranes or after solubilization at various DDM:protein ratios. At a high DDM:protein ratio, rhodopsin could not be cross-linked, a strong indication that it is monomeric and in contradiction with the previous results of Medina et al. At a low DDM:protein ratio, i.e., below the critical micellar concentration (cmc),[5] or in the absence of any detergent, cross-linking induced dimer, trimer, and higher-order oligomer formation as in the work of Medina et al. (*15*). Cross-linking was not complete, and monomeric rhodopsin was still the major species. As mentioned above, it is expected that even a monomeric membrane protein would be cross-linked within retinal rod outer segments because of its very high concentration within the membrane. Simple two-dimensional diffusion of the protein within the plane of the membrane during the 30 min cross-linking would lead to collisional cross-linking. This type of cross-linking certainly occurs with monomeric Ca^{2+}-ATPase within the sarcoplasmic reticulum, and it can only be prevented by reconstitution into phospholipid vesicles at a very high lipid:protein ratio (*37*).

To explain the monomeric species of rhodopsin observed at high DDM concentrations, Jastrzebska et al. (*16*) suggested that putative dimers that pre-exist within the membrane are destroyed by DDM. Although we cannot exclude this possibility, it is only one possibility among many others. For instance, in DDM other oligomeric membrane proteins maintain their quaternary structure, e.g., the photosynthetic reaction center (heterotrimer H,L,M) and the nicotinic acetylcholine receptor channel (pentamer) (*28*, *38*). In more general terms, the state of aggregation in a nonionic detergent is a good indication (although not proof) of the state of the complex in the membrane. Cross-linking in detergent can be a good indication of the oligomeric state in the membrane if the maximum size observed via SDS−PAGE is well-defined. For example, monomers up to pentamers, but no larger oligomers, are detected by SDS−PAGE after cross-linking the native homopentameric channel MscL in either octyl glucoside or Triton X-100 (*39*, *40*).

In a few experiments, Jastrzebska et al. (*16*) used another detergent, CHAPS. Cross-linking prior to membrane solubilization with CHAPS led to the presence of large aggregates as characterized by gel filtration with CHAPS concentrations above the cmc. The size of these aggregates was similar to the size of oligomers formed with DDM below the cmc. On the other hand, in the absence of cross-linking, the size of CHAPS-solubilized rhodopsin as determined by gel filtration in that detergent was comparable to the size of monomeric rhodopsin in the presence of high concentrations of DDM. Surprisingly, Blue Native PAGE analysis of such non-cross-linked membranes solubilized in CHAPS showed massive aggregation, which directly contradicts the gel filtration data in this detergent. However, in this case, Blue Native PAGE was supplemented with concentrations of CHAPS that were

[3] For a membrane protein with a single or small number of α-helical transmembrane segments, the size may be close to the size of the corresponding micelle (*21*, *35*); however, for a protein with 7−11 transmembrane segments, the size may be very different (*35*).

[4] Clearly, 0.97 g/g is likely to be an underestimation of DDM binding; on the basis of our own binding measurements on rhodopsin using other detergents (*27*) and in comparison with several proteins (*28*), one can suggest that ~1.3 g of DDM/g of protein is bound to rhodopsin.

[5] To start the process of solubilization of a membrane, the free concentration of detergent should be close to the cmc or higher (*35*, *45*). Just at the cmc, the membrane proteins are aggregated and only much higher detergent concentrations lead to full solubilization (*35*, *45*). In the experiments described in ref *16*, one can estimate that the total amount of detergent needed to reach full solubilization is in the range of 3 mM DDM.

well below the cmc, which may explain the oligomer formation. Alternatively, CHAPS may favor protein aggregation as has been noted for a related detergent, sodium cholate. McCaslin and Tanford showed long ago (*26*) that rhodopsin solubilized by cholate formed various sized oligomers, the smallest most likely being trimeric and the largest probably being hexameric. Upon exchange of the bound $C_{12}E_8$ with cholate, the monomeric preparation became oligomeric. Similarly, cholate and bile salt derivatives induce self-association of another membrane protein, cytochrome oxidase (*41*). Bovine heart cytochrome *c* oxidase, solubilized by various nonionic detergents, completely dimerizes upon the addition of bile salts (e.g., sodium cholate, deoxycholate, or CHAPS). The relative concentration of the bile salt is critical for the production of homogeneous, dimeric cytochrome oxidase. This protein is never homogeneous and dimeric in detergents such as decyl maltoside, dodecyl maltoside, or Triton X-100. It is homogeneous and monomeric, is a mixture of monomeric and dimeric, or contains higher-order aggregates (*42*). Thus, bile salts induce monomeric cytochrome oxidase to dimerize only under certain well-defined conditions. Some researchers believe that the dimeric form of cytochrome oxidase occurs in vivo, but as stated by Musatov and Robinson (*41*), "its functional role is as yet poorly understood". Bile salts and their derivatives are atypical detergents with a rigid cyclopenthenophenanthrene ring. Furthermore, cholate has a distinctly lower capacity to solubilize membranes or membrane proteins (*43*). The cholesterol-like structure of bile salts and derivatives is very different from that of the alkyl chain detergents, and the latter more closely resemble the structure of phospholipids. Since the rod outer segment membrane notoriously does not contain cholesterol, it seems speculative to draw a firm conclusion from the use of the bile salt derivatives on this protein. The results of the article by Jastrzebska et al. (*16*) are quoted as follows by Park et al. (*9*): "aggregates of rhodopsin have been shown to be differentially preserved depending on the detergent used for extraction". It appears to us that this is a biased interpretation of the data and that a better statement would be "rhodopsin in detergent is always monomeric provided enough detergent is added".

(3) Park and Wells (*17*) studied another class 1a GPCR, the M2 muscarinic cholinergic receptor, which was purified from Sf9 cells. The receptor was extracted in a mixture of digitonin (0.86%) and cholate (0.17%), and the solubilized receptor was purified on DEAE-Sepharose equilibrated in 0.1% digitonin and 0.02% cholate. The complex was not characterized hydrodynamically, but co-immunoprecipitation with two different epitopes was used to assess its oligomeric state. On this basis, Park and Wells state that, "M2 muscarinic receptors appear to coexist as active monomers and largely or wholly inactive oligomers in solubilized extracts" and "solubilization in digitonin/cholate therefore appears to yield a static mixture of monomers and oligomers. The later are inactive". They conclude that, "monomers purified from untreated sf9 cells may pre-exist in the membrane, or they may derive at least in part from the disaggregation of functional oligomers during solubilization". This conclusion is paradoxical: it would imply that mild detergents like digitonin and cholate would disaggregate functional oligomers in active monomers, and preserve nonfunctional oligomers. The pre-existence of functional oligomers is thus a priori postulated, as noted in the first sentence of the abstract: "G protein-coupled receptors are known to form oligomers". It is not supported by any of the data presented in this paper.

(4) Mesnier and Baneres (*18*) used a cross-linking agent to assess the oligomeric state of the leukotriene B(4) receptor BLT1 in the presence of various detergents at concentrations corresponding to their cmc (i.e., in effect, this is a low concentration of detergent; see footnote 4). In a previous study (*44*), the detergent-solubilized inactive receptors were found to be essentially monomeric in DDAO and in DDM. The receptors became dimeric only in the presence of an agonist. Thus, dimerization occurred after detergent solubilization only as a consequence of receptor activation. In this new study, they searched for a detergent that would favor the dimeric state even with an inactive receptor. Of six tested detergents, only one (hexadecyl β-D-maltoside) was found to favor the dimeric state for inactive receptors, but this detergent has an exceptionally long alkyl chain (16 CH_2 groups vs 12 or 13 CH_2 groups for all other detergents that were tested). Such a long alkyl chain makes it a poor solubilizer of hydrophobic membrane proteins; e.g., Ca^{2+}-ATPase is poorly solubilized by this type of detergent (*43*, *45*, *46*). Therefore, this detergent could induce artifactual aggregation of receptors that were monomeric in the membrane. Mesnier and Baneres (*18*) state, "The isolated receptor is essentially a dimer ... provided the detergent used stabilizes the inactive dimeric assembly". Following our previous discussion, this statement could just as well be written, "the isolated receptor is essentially a monomer ... provided the detergent used preserves the monomeric state of the inactive receptor in the membrane".

Structure of the Rhodopsin Monomer in the Membrane and of the Transducin Heterotrimer on the Membrane

As discussed above, the early characterization of rhodopsin as a monomer that interacts functionally with a transducin heterotrimer by forming a transient 1:1 complex has been strengthened rather than invalidated by the recent biochemical studies. But the lack of high-resolution structural data for rhodopsin, and for complete membrane-attached transducin, long precluded the molecular modeling of this complex. High-resolution crystallographic structures became available 10 years ago for a transducin G$t\alpha$GDP−G$t\beta\gamma$ heterotrimer, as well as for the separate Gα and G$t\beta\gamma$ subunits (*47*−*50*). For rhodopsin, a first high-resolution crystallographic structure was published 5 years ago by Palczewski et al. (*51*), and very recently, the group of Li and Schertler much improved the crystallization conditions (*52*) and obtained a structure that fully resolves the conformation of all cytoplasmic loops (*53*).

One first notices that none of the crystallographic studies of rhodopsin suggests the existence of a dimer: no meaningful dimer has been observed in any crystal. In all crystals, the closest protein−protein contacts are between antiparallel rhodopsin molecules, an arrangement that does not make sense for rhodopsin in the native membrane. It is very unlikely that the detergents added for the crystallization process could be responsible for the disruption of protein−protein contacts of a native dimer and the formation of artifactual protein−protein contacts.

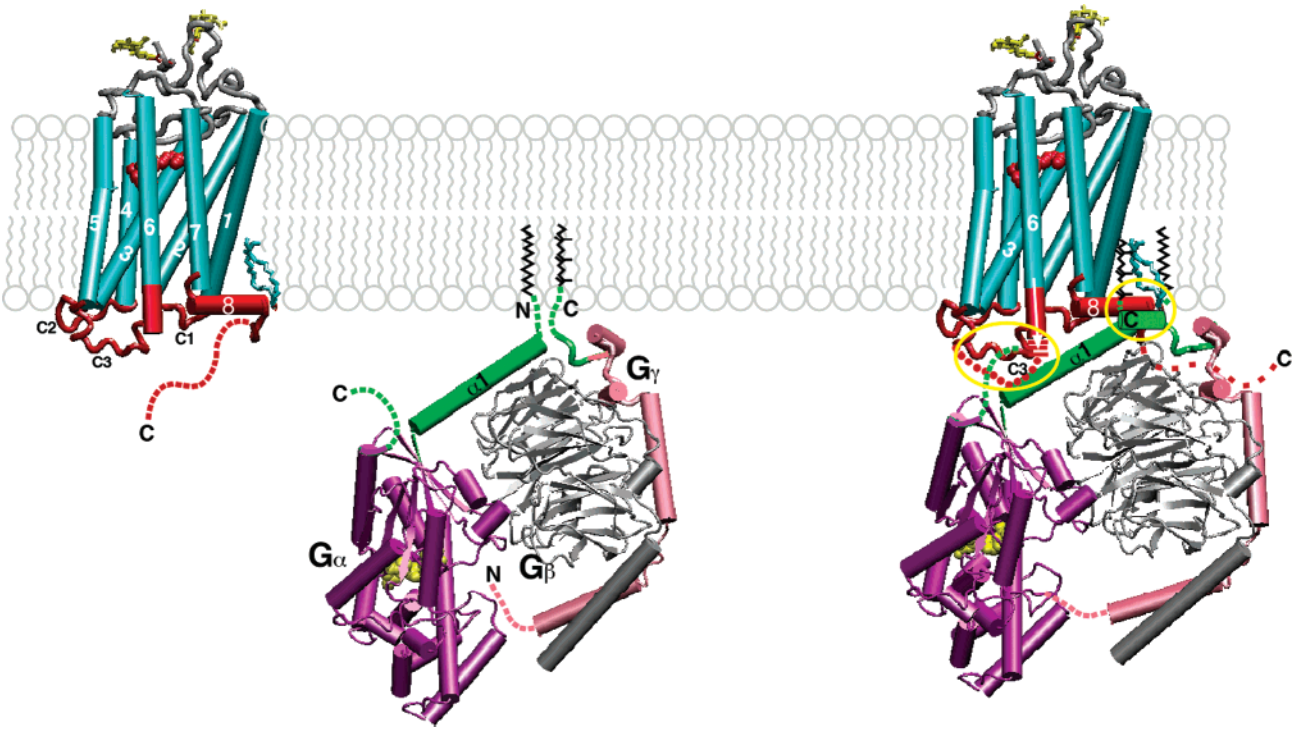

FIGURE 1: Models for a rhodopsin monomer in the lipid membrane, a transducin heterotrimer attached to the lipid membrane, and a complex between a photoactivated rhodopsin and a transducin. The rhodopsin structure is taken from ref *53*; the cytoplasmic loops (c1−c3 and helix 8) that will interact with transducin upon photoactivation are colored red. The C-terminal extension that is present but not resolved in the crystal structure has been drawn as a dashed line. It may be loosely packed on the cytoplasmic loops and may hinder the interaction of inactive rhodopsin with transducin, but upon rhodopsin photoactivation, it will not interfere with or contribute to the interaction of these cytoplasmic loops with transducin. The transducin structure is taken from ref *49*. The N- and C-terminal peptides of Gα and Gγ, which are not present, or present but not resolved in the crystal structure, have been drawn with dashed lines. The segments that are known to interact with photoexcited rhodopsin are colored green. In the rhodopsin−transducin complex model, the main sites of interaction are circled in yellow. The displacement of helix 6 is symbolized by a white arrow and that of cytoplasmic loop c3 by a red dashed line. The loose C-terminal peptide of R* is far from the interaction sites.

One must also keep in mind that these crystallographic structures are incomplete. For rhodopsin, the C-terminal extension is not resolved, past the fourth cytoplasmic loop. Moreover, the structure is that of an inactive rhodopsin which does not interact with transducin. But much information about the conformational change of rhodopsin upon photoactivation has been obtained from extensive mutagenesis and spin labeling studies (*54*−*56*). This concerns essentially helix 6, which protrudes more in the cytoplasm in R* than in inactive rhodopsin, and possibly the C-terminal peptide, which may be loosely packed on the cytoplasmic loops in inactive rhodopsin but does not interfere with or contribute to the interaction of these cytoplasmic loops with transducin upon rhodopsin photoactivation. In the G$\alpha\beta\gamma$ heterotrimer structure (*49*), Gtα lacks both the C-terminal polypeptide, which is critical for the interaction with R*, and the acylated N-terminus, which is critical for interaction with the membrane lipids. Gγ lacks a short C-terminal polypeptide and the farnesyl modification, which inserts in the lipid membrane. These missing terminal peptides and lipid modifications have been added to our molecular model of the membrane-bound Gt$\alpha\beta\gamma$ heterotrimer (Figure 1). They have been positioned in the lipid membrane, according to our own studies of the interaction of native acylated Gtα and farnesylated G$\beta\gamma$ with liposomes (*57*). A different model has recently been proposed by Kisselev (*58*), who suggested that in the isolated membrane-bound Gt$\alpha\beta\gamma$ heterotrimer, the Gγ farnesyl is not in the membrane lipids but is inserted in a hydrophobic cleft between blades 6 and 7 of the Gβ propeller core structure. This hydrophobic cleft has been detected in the structure of G$\beta\gamma$ (*59*) but only when G$\beta\gamma$ is stoichiometrically bound to phosducine, a soluble retinal protein that regulates visual signaling by solubilizing isolated G$\beta\gamma$ from the retinal membrane. The farnesyl-binding hydrophobic cleft cannot be observed in the structure of isolated G$\beta\gamma$, or in that of the inactive Gt$\alpha\beta\gamma$ heterotrimer. Thus, as argued by Lukov et al. (*60*), it must be the binding of phosducin to the Gβ propeller core that induces a conformational change in the propeller, and creates the farnesyl-binding pocket between blades 6 and 7. It is noteworthy that in the absence of phosducine and the presence of membrane lipids, complete farnesylated G$\beta\gamma$ remains attached to the lipid membrane, whereas it becomes soluble upon truncation of the farnesyl (*57*). This strengthens our proposition that in the membrane-attached Gtα−G$\beta\gamma$ complex, as in membrane-attached G$\beta\gamma$, the Gγ farnesyl is inserted in the lipid membrane.

In our model for the membrane-bound Gtα−G$\beta\gamma$ complex, the N-terminal helix of Gtα that supports the Gβ propeller hangs off at a large angle from the membrane plane. This differs from often drawn models (*49*, *61*) that make the implicit assumption that in membrane-attached Gt$\alpha\beta\gamma$, this helix lies on the membrane surface. The exposed face of this helix is very hydrophilic and ionically neutral, with K-D and K-E repetitions, which have no affinity for a phospholipid membrane surface. Only the N-terminal acyl modification is hydrophobic and should be inserted in the membrane.

Molecular Model of the Interaction between a Rhodopsin Monomer and a Transducin Heterotrimer

In their plea against a functional interaction between a transducin heterotrimer and a rhodopsin monomer, Park et al. (*9*) argue that "the photoreceptor heterotrimeric G-protein transducin Gt is larger than a monomeric rhodopsin" and claim that one Gt$\alpha\beta\gamma$ must interact with two rhodopsins, one making contact with the Gtα subunit and the second with the G$\beta\gamma$ subunit. But the various molecular models in support of this hypothesis are built with a rhodopsin in the inactive state conformation and a transducin heterotrimer in which Gtα lacks both its N-terminal acyl modification and its C-terminal decapetide, and Gγ lacks its C-terminal farnesyl. In these models, the transducin heterotrimer has no contact with the membrane lipids. This weakens the value of arguments based on the relative sizes of the interacting surfaces of rhodopsin and transducin.

The C-terminal peptide of Gtα was early recognized as a critical motif of the interaction of the Gtα−G$\beta\gamma$ complex with photoactivated rhodopsin (R*), through the well-known decoupling effect of pertussis toxin, which ADP-ribosylates Gtα specifically on a cysteine residue near its C-terminus. The binding site of the Gtα C-terminal peptide in R* has been precisely located near the cytoplasmic end of helix 6 of R* (*62*). That the farnesylated C-terminal domain Gγ is also involved in the coupling of Gt$\alpha\beta\gamma$ to R* was recently demonstrated by Kisselev et al. (*63*), who observed that the Gtγ(60−71)-farnesyl peptide could stabilize the activated Meta II state of rhodopsin, which was independent of the similar effect observed with the C-terminal peptide of Gtα. Kisselev (*58*) further observed by NMR that this Gtγ(60−71)-farnesyl peptide, which remains unstructured in the presence of inactive receptors, forms an amphipatic helix upon rhodopsin activation. Thus, both the Gtα and Gtγ C-terminal peptides, which do not interact with inactive rhodopsin, interact with photoactivated rhodopsin, at two different sites.

In the various docking models of a rhodopsin dimer to transducin (*11*−*13*), the two sites for Gtα and for Gt$\beta\gamma$ are proposed to be on the two adjacent rhodopsin molecules that form the dimer. These models thus require that both rhodopsins in the dimer be simultaneously activated. This is not realistic in a retinal rod which responds reproducibly to a single photon (activating necessarily a single rhodopsin molecule) and whose physiological response range corresponds to excitation of one rhodopsin in hundreds of thousands. Under such conditions, the probability that two contiguous rhodopsins would be simultaneously photoexcited is nil. Thus, the two sites for the Gtα C-terminal peptide and for the Gtγ C-terminal farnesyl peptide must be on a single R*. Contrary to what is assumed by the dimer proponents, this is fully compatible with the structure and size of rhodopsin and of transducin, provided they are properly positioned in and on the lipid membrane, respectively. The main motifs of Gt$\alpha\beta\gamma$ that interact with R* are the C-terminal peptide of Gtα, helix α1 of Gtα, which is close to its N-terminus, and the C-terminal peptides of Gtγ. The two C-terminal peptides of Gtα and Gtγ are not visible on the crystallographic structure of the truncated soluble heterotrimer, but they are located near the two extremities of N-terminal helix α1 of Gtα. There is no steric problem to have both these motifs interacting with the same R*.

As an addition to many existing models of the "one receptor−one G-protein" type, we propose a model (Figure 1) in which the C-terminal peptide of Gtα contacts the third cytoplasmic loop of R* near the end of helix 6 that protrudes in the cytoplasm, and is more prominent in R* than in inactive rhodopsin (*54*, *56*). The C-terminal peptide of Gtγ, which folds in an amphipatic helix upon binding to R* (*58*), must contact R* at a site that is sufficiently distant from helix 6 and is near the interface with the lipid membrane in which the farnesyl remains inserted. We suggest that this binding site is along cytoplasmic helix H8 of R*, which has been shown to be directly involved in the rhodopsin−transducin interaction (*64*, *65*). This positions on the cytoplasmic surface of R* helix α1 of Gtα, which in the heterotrimer structure is located between the Gtα and Gtγ C-terminal peptides. As proposed by Yeagle and Albert (*66*), this α1 helix is constrained to make all along its length close contacts with the three cytoplasmic loops of R*. The acyl modification at its N-terminus is still inserted within the lipid membrane, possibly close to the palmitoyl chains downstream of helix 8 of R*. The C-terminal extension of R* does not seem to interfere or contribute to the interaction of R* with transducin. It is probably unfolded away from the cytoplasmic loops.

This is just one example of a model that is fully compatible with monomeric rhodopsin. The lack of high-resolution structural data on rhodopsin in the activated state limits the attempts at more precise molecular docking of Gt$\alpha\beta\gamma$ on rhodopsin, but this low-resolution model provides a hint for a mechanism that has been proposed for the activation of the Gt$\alpha\beta\gamma$ heterotrimer by R*: the constraint of the cytoplasmic loops of R* on helix α1 of the bound Gtα could induce a lever-arm motion of this α1 helix with respect to the Gtα core domain (*67*, *68*). As helix α1 of Gtα supports the Gβ propeller domain, this would induce displacement of G$\beta\gamma$ with respect to Gtα, closely packing the Gtα−Gβ interface and creating a novel interface between Gtα and the N-terminus of Gγ, which would induce the release of GDP in Gtα (*68*).

This model could be valid for all GPCRs, since they all share conserved motifs in the cytoplasmic loops that bind the heterotrimeric G-protein. It is important to note that this validity is not dependent on the fact that some GPCRs may exist as constitutive dimers. We have seen that this is not the case for the rhodopsin-like class 1a GPCRs that are structurally monomeric in the inactive state; however, the class 3 GPCRs are constitutive homo- or heterodimers, and their 7TM domains couple individually to their cognate G-protein (*8*). Strikingly, when it has been deleted of its N-terminal and C-terminal domains, the expressed 7TM domain of a GABA receptor does not form dimers anymore, but is still able to activate G-proteins. Thus, with regard to their signaling functions that depend on their coupling to heterotrimeric G-proteins, all GPCRs appear to act as momomers.

Future Studies: High-Resolution Structure of the R−Gt$\alpha\beta\gamma$ Complex between a Rhodopsin Monomer and a Nucleotide-Depleted Transducin Heterotrimer*

The poor stability of photoactivated rhodopsin in the native membrane, and even more so when it is solubilized in

detergent, makes it highly improbable that one would obtain crystals of photoactivated rhodopsin, but the high-affinity stoichiometric complex between R* and nucleotide-free Gt$\alpha\beta\gamma$, which can be obtained upon illumination of retinal rod membranes in the absence of GTP, has proven to be very stable (*32*) and appears to remain stable upon detergent solubilization. Crystallization of such a complex could provide in a not too distant future the first high-resolution picture of an activated GPCR monomer interacting with a heterotrimeric G-protein.

There is still a bright future for monomeric rhodopsin and class 1a GPCRs.

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