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REVIEW ARTICLE

Photo-affinity labeling strategy to study the binding site of G protein-coupled receptors

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G protein-coupled receptors (GPCRs) are involved in the control of every aspect of our behavior and physiology. GPCR can be involved in pathological processes as well and are linked to numerous diseases, including cardiovascular and mental disorders, retinal degeneration, cancer, and AIDS. This article reviews the methods of approaching photo-affinity labeling strategy to obtain the possible G protein-coupled receptors's binding site.

Keywords GPCR, photo-affinity, labeling, rhodopsin, transmembrane, structure, binding

1 Introduction

G protein-coupled receptors (GPCRs) form one of the most versatile families of proteins and respond to a chemical diversity of signal transduction molecules. GPCRs are the largest family of membrane proteins and they are also closely related to hormones and neurotransmitters, such as vision, olfaction and taste [1]. In recent years, there are remarkable progresses in the structural biology of GPCRs. The crystal structures of lots of GPCRs are solved, including opsin that is an active form of rhodopsin. All of these have made great contributions to membrane-protein biophysics, cell biology, physiology and drug discovery [2].

Many top-selling drugs from the past and present target membrane-bound GPCRs, and the pipelines of most pharmaceutical industries are filled with GPCR-targeting molecules. G protein-coupled receptors are involved in the control of every aspect of our behavior and physiology. This is the largest class of receptors, with several hundred GPCRs identified thus far. GPCRs regulate some part of nearly all physiologic functions. There are more than 800 known GPCRs in the human genome. Ligands for these receptors include large glycoprotein hormones, a multitude of peptides, bioactive lipids, amino acids and amino acid metabolites such as dopamine and norepinephrine, and small molecules such as acetylcholine and sucrose [3]. G protein-coupled receptors can be involved in pathological processes as well and are linked to numerous diseases, including cardiovascular and mental disorders, retinal degeneration, cancer, and AIDS [4,5]. The Rhodopsin receptor family is the largest family of GPCRs and contains around 670 full-length human receptor proteins [1]. There are four groups — α, β, γ and δ subunits comprising this family. The olfactory receptor is the largest cluster of members and is in the δ group [6]. The Rhodopsin family of GPCRs is highly heterogeneous when the structure is maintained, and meanwhile, the diversity is not found in their N termini, where most receptors have only a short stretch of amino acids. Activated receptors (R*) can then activate heterotrimeric G proteins (composed of α, GDP, β and γ subunits) on the inner surface of the cell membrane [7–9]. In 2000, Palczewski and colleagues presented the first crystalized high-resolution structure of a GPCR: the bovine rhodopsin model [10]. This study showed that the seven helices are arranged in an anticlockwise manner when seen from the extracellular side of the membrane [11]. Because the incorporation of ligand information from related receptors can provide a pharmacophore based approach to optimize high-throughput screening [12], a plenty of structure-based design will take root in either high-quality three-dimensional computational model or the crystal structure of the ligand pocket of the GPCR [13].

All of these applications have brought out such questions as how GPCRs activate G proteins and cause such specific responses in cells, what are the triggering changes in GPCRs on agonist binding and how they fold, and what causes misfolding in so many genetic diseases, which are all the hottest topics in the scientific world.

2 Photo-affinity labeling strategy

Photo-affinity labeling has evolved in recent 30 years, although the basic principle remains unchanged since late 60s [14,15]. Photo-affinity labeling requires the use of a photoactivatable but chemically inert ligand analog, which could be activated by the light and produces a highly reactive species that will bind irreversibly to the biologic receptor at the site of interaction [16–18]. Upon irradiation of the resulting ligand-receptor complex, a reactive intermediate will bond covalently to the receptor proximal to the binding site of the ligand. There are a lot of examples that these photolabeled ligands allowed the characterization of functional proteins on sodium dodecylsulfate (SDS) gels. However, only a few of these experiments led to the

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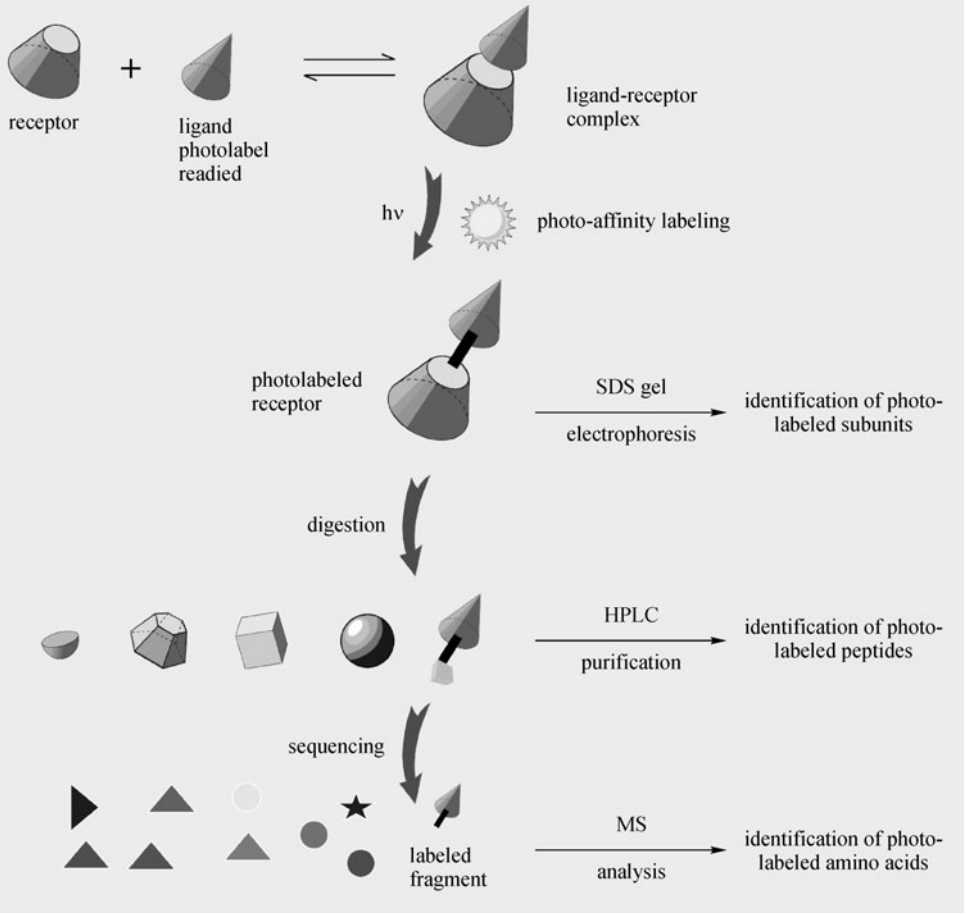
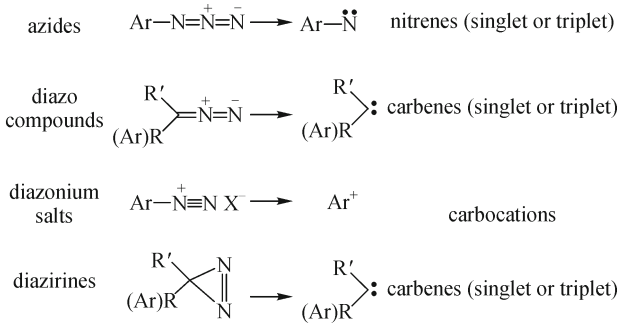


Figure 1 Photo-affinity labeling principle and the steps to identify the fine structure information

identification of the amino acid residues labeled by the photosensitive probes, which will require further process, such as digestion or purification to obtain much more detailed fragments to reach the analysis requirement. Following photo-crosslinking, receptor digestion is used to generate several unmodified peptides among which one is labeled with the photolabile ligand. This ultimate step gives important structural information, the position of the binding site on the primary structure of a receptor and precise data on the ligand-receptor interaction. (Fig. 1) Analysis of the labeled receptor fragment can be performed by using HPLC as a separation technique coupled with online detection of the labeled fragment using liquid scintillation counting [19], fluorescence detection [20], or identification of mass spectrometric tags [21].

There are a few chemical functions that could be used in photo-affinity labeling experiment. Photochemical fragmentations that induce the release of nitrogen becomes the most widely used photochemical groups because nitrogen is an inert gas and several chemical functions can serve as precursors, such as azido, diazo, and azo groups, diazirines, and diazonium ions. These functional groups serve as precursors of reactive species such as nitrenes, carbenes, radicals, and carbocations. (Scheme 1)



Scheme 1 Several photo-affinity labeling groups used as precursors

Aryl azides are the most frequently used photo-affinity labels among those groups, and it also includes azidonucleotides and fluoriated azyl azides. The former is usually used as excellent nucleotide analogs with a minimum of chemical modification since the azido group is incorporated on the

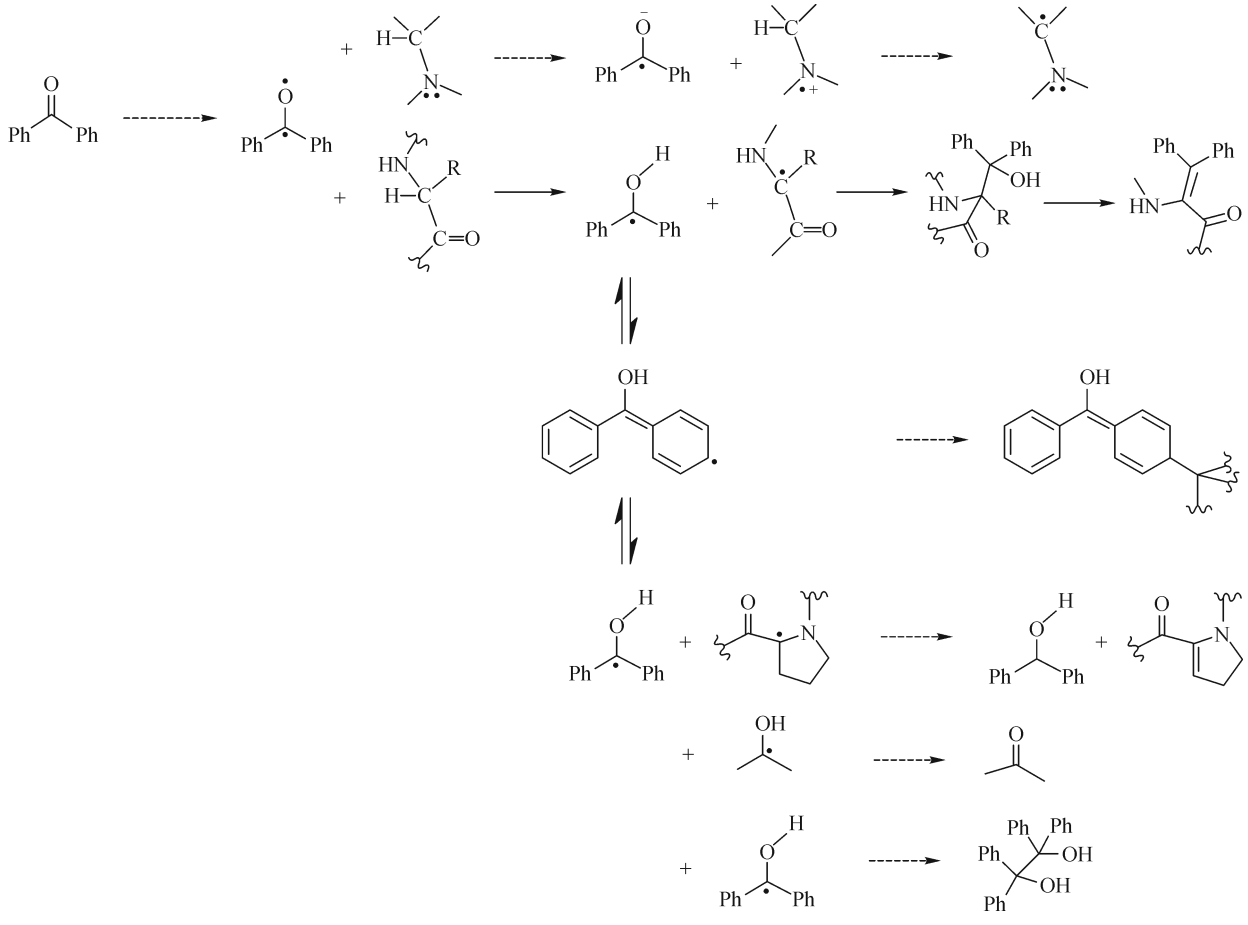
heterocyclic ring [22–30]. The latter has made up by far the most promising development in the aryl azide series for photo-affinity labeling. They have benefited from singlet fluorinated arylnitrene which rearranges more slowly to the corresponding didehydroazepine than the unsubstituted species, thus allowing this intermediate to react with most chemical bonds [31–35]. However, fluorinated aryl azides can be activated only by light with wavelengths less than 300 nm, which might be unfavorable for sensitive proteins.

In addition to the aryl azides, a great variety of other photosensitive compounds have been described as potential probes for photo-affinity labeling [36]. Aryldiazonium salts have electron-withdrawing substituents, which are unstable species under nonacidic conditions. But in ortho and para position they are conferred sufficient stability and also interesting photochemical properties such as high quantum yields [37] and absorption at wavelengths greater than 300 nm, which makes them a good photo-affinity labeling group; Aryldiazirine derivatives, especially trifluoromethylaryldiazirines, show favorable photochemical properties in addition to excellent chemical stability. When irradiated, the diazirine either undergoes fragmentation to the corresponding carbene or isomerizes to a linear diazo compound [38].

Benzophenones have been used extensively for photo-affinity labeling experiments, and are typical examples of precursors of photogenerated radicals. Basically, Benzophenones are chemically more stable than diazo esters, aryl azides, and diazirines, and they can be manipulated in ambient light and can be activated at 350–360 nm. Lastly, and the most significantly, benzophenones can even react with unreactive C-H bonds, even in the presence of solvent water and bulk nucleophiles [39]. These three properties combine to produce highly efficient covalent modifications of macromolecules, frequently with remarkable site specificity. The physicochemical properties of benzophenones is that the reactive triplet state does not rearrange, and can be easily generated at long wavelengths, avoiding significant damage to aromatic amino acids of proteins and is able to be inserted into C-H bonds [39]. (Scheme 2)

3 The unknown binding site between G protein and rhodopsin

Rhodopsin is the best known GPCR, so it gives us an



Scheme 2 Photochemistry of benzopheone chromophore and redical recombination pathways

opportunity to find the mechanisms of how GPCRs activate G proteins and cause such specific responses in cells and where the triggering changes in GPCRs on agonist binding. Binding of extracellular ligands causes a conformational change in the GPCR to enable its cytoplasmic domain to catalyze GDP/GTP exchange in heterotrimeric G proteins (Gαβγ), the process that initiates the signaling cascade. Visual signal transduction in the retinal rod cell is a prototypical sample of G protein-coupled signaling systems in which the GPCR rhodopsin, with its covalently bound chromophore 11-*cis*-retinal, acts as a photoreceptor to detect single photons. In its photochemically functional core, rhodopsin contains the covalently bound ligand 11-*cis*-retinal, which stabilizes the inactive rhodopsin state. Photon absorption causes retinal *cis-trans* isomerization, leading to in situ formation of an activating ligand in the binding site and subsequent activation of rhodopsin [40].

Visual transduction is the process in which visual cells convert light into a neural signal, which in turn is transmitted to the brain via the optic nerve. As we know, during 11-*cis* → *trans* isomerization of the chromophore in rhodopsin, there is a sequential thermal relaxation of the pigment through the intermediates: photo-Rh (λ_{max} 555 nm) → batho- (–140°C, 535nm) → lumi- (–40°C, 497 nm) → meta-I- (–15°C, 480 nm) → meta-II-Rh (0°C, 380 nm). Except for photo-Rh, the intermediates can be monitored at the temperatures indicated and have been submitted to various spectroscopic and other studies [41]. The shape of the protein at the meta-II-Rh stage initiates a cascade of enzymatic reactions, starting with catalysis of nucleotide exchange (guanosine diphosphate replaced by guanosine triphosphate) in more than 1000 molecules of G protein (called transducin in vision) per second, which, via the activation of phosphodiesterase, results in hydrolysis of 100,000 molecules of cyclic guanosine monophosphate (cGMP). This drop in cGMP closes the cation-specific channels in the rod outer segment of visual cells and leads to a build-up of electric potential resulting in generation of the neural signal [42–47].

Recent progress in structure determination of both Gt (transducin) and rhodopsin reveals a wealth of information about how retinal is bound and how the rhodopsin ground state is stabilized. It also shows that critical residues for G protein activation (E134, R135) are buried and inaccessible to the rod photoreceptor G protein and transducin [48]. But the molecular mechanism of signal transfer between these two proteins is not clear. Several putative interaction sites on the G protein have been reported, but only two of them (shown in orange), the Ga C-terminal tail (CTa, residues 340–350) and the farnesylated C-terminal tail of Gg (CTg-far, residues 60–71), have been "confirmed" to specifically bind and stabilize the active rhodopsin conformation [21,49]. Likewise, binding surfaces involve the cytoplasmic (C-terminal side) loops of rhodopsin and domains of Gtα- and Gtγ-subunits [8,50–52]. The α helix of transducin [53] have showed that some amino acids from the second and third loops may be the determinants in binding of Gt and release of GDP from Gtα. Gtα (340–350) (IKENLKDCGLF) and Gtγ (60–71) farnesyl (DKNPFKELKGGC-farnesyl) have been found to mimick the behavior of the Gt holoprotein in terms of meta-II stabilization and blockage of rhodopsin photoregeneration to the groundstate [21]. Fig. 2 shows the relative positions of the two C-terminal peptides in the X-ray structure of GDP bound form of transducin [54].

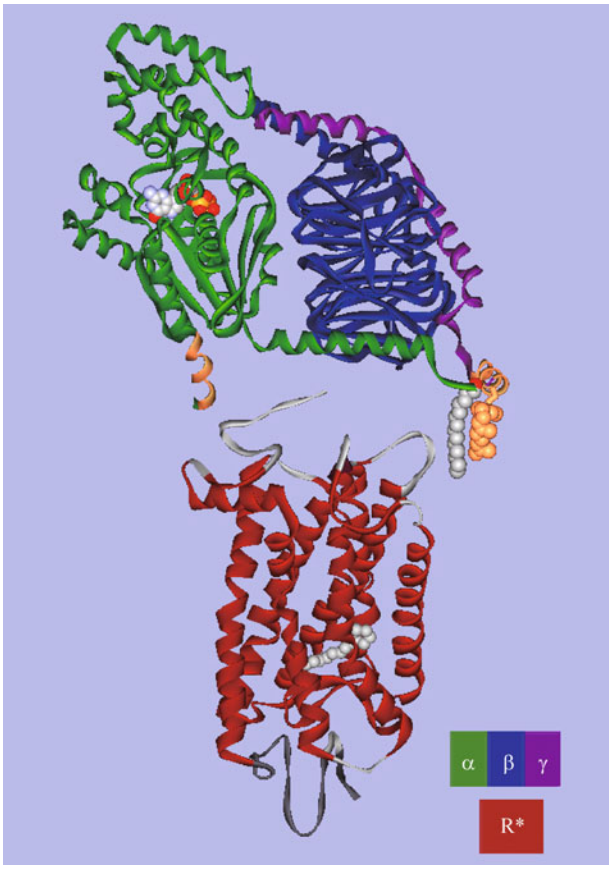


Figure 2 Interface between the heterotrimeric G protein transducin and its receptor rhodopsin. Transducin (1GOT) consists of the nucleotide binding Gtα subunit and the heterodimeric Gtβγ subunit binding sites for transducin on the GPCR rhodopsin (1HL9), which contains the bound chromophore 11-*cis*-retinal (*gray*, space-filling model).

These peptides compete with Gt for the active rhodopsin receptor in the micromolar range and, in high enough concentrations, can replace part of the G protein in direct activation assays [21]. Cai and coworkers used particular cysteines on rhodopsin's cytoplasmic side to crosslink transducin using two different heterobifunctional crosslinking reagents, one photoactivatable and the other chemically

preactivated [55]. They could crosslink transducin from a variety of sites on the second and third intracellular loops with each of these reagents, consistent with current expectations that these loops are involved in G protein binding. In the case where the crosslinkers derivatized Cys-240 on the third intracellular loop, the sites on transducin that had been crosslinked were analyzed by mass spectroscopy to identify the insertion site. This pocket of hydrophobic interactions couples the amino and carboxyl-terminal regions of Gα. However, little is known structurally about the bound conformation of the Gtγ (60–71) far peptide or its binding site on light activated rhodopsin, especially with regard to its absolute lipid requirement for recruitment to the disc membrane [56–58]. The photolabile rhodopsin mutant was crosslinked to transducin while solubilized in detergent micelles and immobilized on a 1D4 antibody column which may have significantly lowered its affinity toward Gtγ containing the hydrophobic farnesyl moiety [55]. Indeed, Hofmann and coworkers found that increasing the dodecyl maltoside concentration dramatically reduced binding of Gtγ (50–71) far but did not change light activated rhodopsin's affinity for Gtα (340–350) [43].

Recently, Chen and coworkers used a photo-reactive group to attach the Gtγ peptide and to perform the photocrosslink with the cytoplasmic surface of rhodopsin, thereby a photolabeling study could be carried out using light activated rhodopsin in its native disc membrane where Gtγ (50–71)far is known to have the highest affinity. By analyzing the result of the photocrosslink, they have almost obtained the answer where the binding site between G protein and GPCR is [59].

4 Summary

Usually, the techniques that have been used to locate the active sites of enzymes or the binding sites of ligand-receptor pairs on a molecular level is X-ray crystallography of the complex, which perhaps gives the most information (i.e. 3D arrangements of atoms and multiple contact points). However, such data are difficult to obtain, especially with complexes involving integral membrane proteins like the present example, GPCRs. As an alternative, photo-affinity labeling has been explored and has shown a promising result.

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