

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/263974821>

# Probing G Protein–Coupled Receptor—Ligand Interactions with Targeted Photoactivatable Cross–Linkers

ARTICLE *in* BIOCHEMISTRY · NOVEMBER 2013

Impact Factor: 3.02 · DOI: 10.1021/bi401300y

---

CITATIONS

7

---

READS

20

2 AUTHORS, INCLUDING:



Thomas Sakmar

The Rockefeller University

218 PUBLICATIONS 10,843 CITATIONS

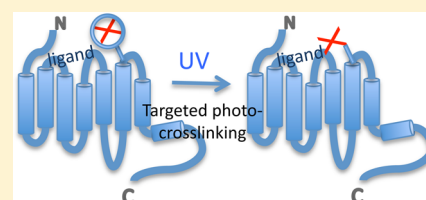
SEE PROFILE

# Probing G Protein-Coupled Receptor—Ligand Interactions with Targeted Photoactivatable Cross-Linkers

Amy Grunbeck and Thomas P. Sakmar\*

Laboratory of Chemical Biology and Signal Transduction, The Rockefeller University, 1230 York Avenue, New York, New York 10065, United States

**ABSTRACT:** It has been 50 years since F. H. Westheimer and colleagues reported the first use of a photoactivatable cross-linking reagent to study the active site of chymotrypsin. In studies of seven transmembrane helical receptors, also known as G protein-coupled receptors (GPCRs), recent simultaneous advances in structural biology, molecular dynamics simulations, and amber codon suppression methods have allowed the development of a targeted photo-cross-linking strategy to probe receptor–ligand interactions in cell membranes. We review here recent advances in targeted photo-cross-linking of GPCR–ligand complexes in the context of extensive earlier work that primarily relied upon the use of ligand analogues with photoactivatable constituents.



Membrane proteins with seven transmembrane (TM) helical segments (7-TM receptors) are also commonly referred to as G protein-coupled receptors (GPCRs) because they interact with cytoplasmic guanine nucleotide regulatory proteins (G proteins) in a canonical signal-transduction pathway. A superfamily of related 7-TM receptors comprises the largest family of cell surface receptors in humans, and 7-TM receptors are found ubiquitously in eukaryotic organisms. Since 7-TM receptors bind to hormones, cytokines, metabolites, neurotransmitters, neuromodulators, and other signaling molecules, they have been frequent targets of drug discovery programs. Up to one-third of all human molecular drug targets are 7-TM receptors. Despite extensive advances in studies of 7-TM receptors from a variety of overlapping scientific research disciplines, including molecular pharmacology, structural biology, and molecular dynamics simulations, how the binding of a ligand on the extracellular side of a receptor causes the release of guanosine 5'-diphosphate (GDP) from a G protein bound to the cytoplasmic side of the receptor—a distance of ~80 Å—is not understood with chemical precision. One tool that continues to advance our understanding of GPCR–ligand interactions and circumvents some of the obstacles of working with these complexes is the application of photoactivatable cross-linkers.

Photoactivatable cross-linking reagents have been used to study binding interactions within biological systems since the 1960s. Westheimer and colleagues pioneered the early work by applying photoreactive molecules to map an enzyme's active site.<sup>1</sup> One of the advantages of using photoactivatable probes in a biological system is that probes react instantaneously with surrounding chemical bonds upon photolysis.<sup>2</sup> The ability to photoactivate a cross-linker reagent allows, at least in principle, for control over “when and where” a cross-linking reaction takes place. Therefore, these reagents enable the investigation of the structures of transient complexes in a cellular environment, for example, which is not possible with standard chemical or crystallographic techniques. Most physiologically

relevant GPCR–ligand complexes require the native plasma membrane environment for complex formation. Photolabile probes with high affinity for receptors under native conditions and with minimal alteration to the structure of the original ligand or of the ligand–receptor complex are required to gain maximal information.

Several different reagents are reactive upon photolysis, but only a handful of these are stable within a biological environment. A few of the most common ones used to study biological interactions include diazoacyl ester,<sup>1</sup> aryl azide,<sup>5</sup> benzophenone,<sup>6</sup> and diazirine.<sup>7</sup> Each of these molecules has its own inherent advantages and disadvantages, which have been described in detail elsewhere.<sup>8,9</sup> The primary characteristics that differ among these molecules and determine their effectiveness to probe biological interactions are size, reactivity, and stability. Over the past 40 years or so photo-cross-linking reagents have been popular tools for the study of GPCR–ligand interactions. The two obvious general strategies for investigating GPCR–ligand interactions with chemical cross-linkers are by attaching the photoactivatable moiety either to the ligand or to the receptor (Figure 1). Here we review not only the methodologies that have been used to generate photoactivatable GPCR ligands but also the recent advances in the site-specific incorporation of photoactivatable cross-linkers into GPCRs expressed in mammalian cells, a method known as targeted photo-cross-linking.

## ■ PHOTOACTIVATABLE GPCR LIGANDS

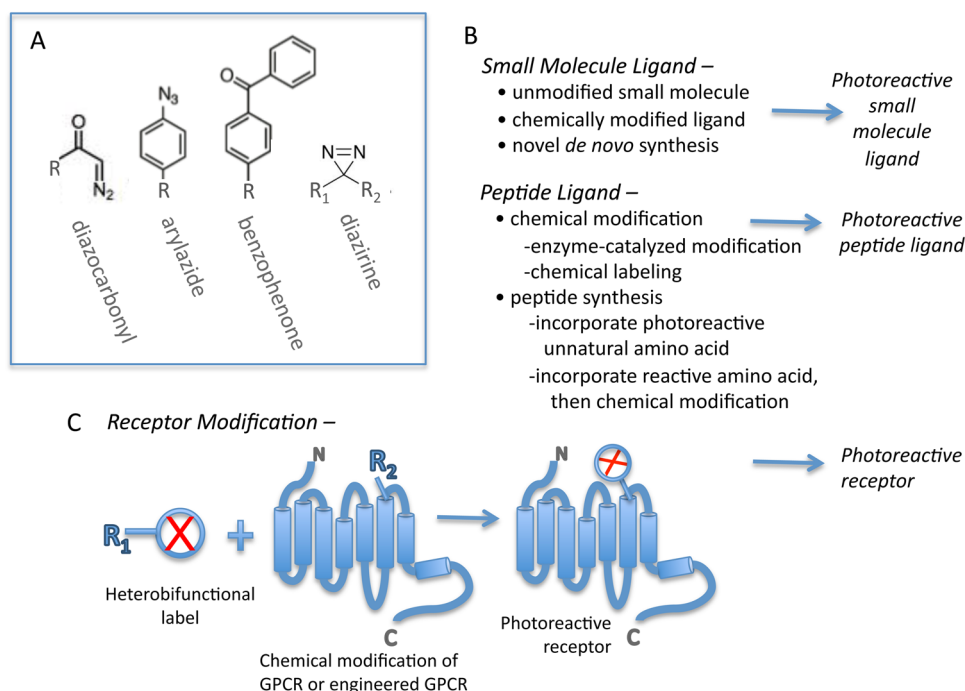
Photoaffinity labeling is a potentially powerful technique for identifying the receptors for orphan ligands and for determining a ligand binding site on a receptor. To perform these studies an analogous ligand needs to be designed that contains a

**Received:** September 18, 2013

**Revised:** November 6, 2013

**Published:** November 7, 2013





**Figure 1.** Strategies to incorporate photoreactive moieties into ligands and receptors. (A) The most commonly used photoreactive moieties used in mapping ligand binding sites in GPCRs are shown. These groups have been used to study GPCR–ligand interactions by incorporating them either into the ligand or into the receptor. (B) Strategies are outlined to create photoreactive small molecule ligands or photoreactive peptide ligands. Some native small molecule ligands naturally contain photoreactive groups. Other photoreactive small molecule ligands are generated by chemically labeling a molecule with a hetero-bifunctional group or by synthesizing a photoactivatable analogue *de novo*. Peptide ligands are labeled postsynthesis through an enzyme-catalyzed reaction or chemical modification. Photoreactive peptide ligands are also created by direct synthesis using natural amino acids derivatized with photo-cross-linking groups or photoactivatable unnatural amino acids (UAAs). (C) Photoreactive groups can also be linked to receptors using traditional chemical modification approaches, typically at a reactive cysteine residue that exists in the native structure or is introduced by site-directed mutagenesis. Expressed receptors are generally purified from cell extracts before modification. The approaches described in B and C are all limited because modification of the ligand or receptor by the introduction of the photoreactive group might affect function. The effects of ligand or receptor modifications may be difficult to predict in advance.

photoactivatable cross-linking moiety while retaining binding affinity to the cognate receptor. Photoaffinity ligands have been synthesized in several different ways depending on whether the ligand is a small molecule, a peptide, or a protein (Figure 1). In the sections below we describe a few examples of how photoactivatable GPCR ligands have been generated.

**Synthetic Small Molecule Ligand Analogues.** Many of the native ligands for family A, or rhodopsin-like GPCRs, are small molecules. For example, the prototypical GPCR rhodopsin serves as the dim-light photoreceptor in the rod cells and binds the chromophore 11-*cis*-retinal, which acts like a pharmacological inverse agonist. To create an analogue of a small molecule that contains a photogenerated cross-linker generally requires extensive organic synthesis, as was the case in studies to determine the binding site and orientation of 11-*cis*-retinal in the transmembrane core of rhodopsin. Several analogues of 11-*cis*-retinal were synthesized to contain a diazirine group as a substituent on the  $\beta$ -ionone ring.<sup>4</sup> These molecules were characterized for their ability to regenerate opsin. Only one of the analogues was able to bind to opsin to regenerate a pigment and found to cross-link to opsin upon UV exposure. After further analysis the orientation of 11-*cis*-retinal was determined to be such that the  $\beta$ -ionone ring was directed toward TM helices 3 and 6.<sup>5</sup> Cross-linking an 11-*cis*-retinal analogue to opsin is a classic example of how it is possible to retain binding of a small molecule ligand to its receptor after introducing a photo-cross-linking group. In addition, this work demonstrates the manner in which synthetic ligand analogues

can determine the absolute orientation of a molecule in the ligand binding site of a receptor without either extensive site-directed mutagenesis and ligand binding assays on receptor mutants or a high-resolution crystal structure of a receptor–ligand complex.

Rhodopsin is unique compared to many other GPCRs because it is found in large abundance within the disc membrane of the retinal rod cell and can be purified to homogeneity in detergent solution. Other receptors are expressed at much lower levels in the native tissues, which complicates their identification, isolation, and characterization. In such cases, the development of photoaffinity ligands is advantageous to identify target receptors and to probe ligand specificity. One particular demonstrative example is the identification of the sex pheromone receptor in the silk moth *Antheraea polyphemus*. Ganjian et al. synthesized a diazoacetate derivative of the sex pheromone (6*E*,11*Z*)-6,11-hexadecadienyl acetate (HDA) and demonstrated that this molecule retained 10% of the native activity of the pheromone.<sup>10</sup> UV irradiation of the diazoacetate HDA analogue when bound to sensory hairs from the silk worm resulted in the identification of a 69-kDa membrane protein.<sup>11</sup> Prior to this discovery the involvement of a membrane receptor in the sex pheromone transduction mechanism was only a hypothesis.

**Native or Native-like Ligands.** Family A GPCRs are the single largest target class for commercially available pharmaceutical agents. Approximately 22% of molecular drug targets are rhodopsin-like GPCRs, far outweighing the next biggest

class, ion channels.<sup>12</sup> Pharmacological and biochemical characterization of target receptors has generally been a prerequisite for effective drug development, so it is no surprise that some of the most important GPCR drug targets have been studied extensively by photo-cross-linking methods. In some cases, native ligands are able to cross-link without the addition of specific reactive functional groups. For example, dopamine is an example of a native photoaffinity ligand. Nishikori et al. were the first to discover that UV illumination of mammalian nervous tissues in the presence of dopamine led to the covalent attachment of dopamine to specific cellular proteins. Dopamine does not contain one of the common photoactivatable cross-linking groups, and this report proposed that the photocross-link occurs between the oxygen in the catechol moiety of dopamine and an amino acid residue in the dopamine receptor.<sup>13</sup> Through the use of dopamine as a photoaffinity probe, Nishikori and colleagues were also able to apply <sup>3</sup>H-dopamine to quantify the amount of purified receptor and identify that the D1 and D2 dopamine receptors are coexpressed in mammalian nervous tissues.<sup>13</sup> Others followed up on this study and used the photoactive properties of dopamine to determine that dopamine binds specifically to a 57-kDa protein subunit in striatal membranes that they suggested was the D1 dopamine receptor.<sup>14</sup> There are many advantages to performing photo-cross-linking studies when the native structure of the ligand is retained, especially with small molecule ligands in which addition of even a small chemical group significantly alters the structure of the molecule.

Some drugs are themselves photoactivatable cross-linkers without further modification, although efficiency of the photolysis and cross-linking can be very low. Members of the adrenergic receptor subfamily of GPCRs have been extremely popular drug targets because of their significance in regulating the cardiac, pulmonary, and circulatory systems. For example, the  $\beta$ -adrenergic receptors have been the focus of many photo-cross-linking studies involving photoreactive drug analogues. Isoprenaline, a  $\beta$ -adrenergic receptor agonist used as a cardiac inotropic agent and pressor substance, is one example of a GPCR drug that has been shown to label covalently a receptor upon photolysis without further modification of the molecule.<sup>15</sup> Other  $\beta$ -adrenergic receptor antagonists, generally termed  $\beta$ -blockers, have required alternative syntheses to incorporate a photoreactive group, resulting in the development of a plethora of  $\beta$ -adrenergic azide-<sup>16–21</sup> and diazirine-containing probes.<sup>22</sup> Obviously, the ideal probe ligand contains a photoreactive moiety at a location distinct from its pharmacophore so that pharmacological activity is maintained. Such synthetic ligand tools have been useful for the study of the  $\beta$ -adrenergic and other types of related receptors and exemplify how a variety of different photo-cross-linkers based on small molecule analogues of GPCR ligands can be used to study ligand binding sites.

**Derivatizing Peptide Ligands with Photoactivatable Groups.** A large number of GPCRs are activated by endogenous peptide hormones, neuropeptides, or peptide-derived ligands (peptides that are post-translationally modified). Peptide ligands that target GPCRs have been labeled with photo-cross-linking groups postsynthetically either through enzyme-catalyzed reactions or through chemical modifications. The application of modifying enzymes for labeling purposes provides unique specificity and allows for labeling at positions that cannot generally be targeted with chemical reagents. For example, Gorman et al. demonstrated the use of guinea pig liver transglutaminase for the attachment of aryl azides onto the  $\gamma$ -

carboxamide of glutamine residues in peptide ligands. Novel substrates for transglutaminase were synthesized and then reacted with peptide ligands, such as substance P and glucagon 1–6.<sup>23</sup> This technique was also applied to generate a photosensitive calcitonin analogue using the transglutaminase substrate *N*-( $\beta$ -aminoethyl)-4-azido-2-nitroaniline. Photolysis of this calcitonin analogue when bound to T47D breast cancer cells led to the identification of an 85-kDa calcitonin binding protein.<sup>24</sup> The photoaffinity probe in this example facilitated the identification of the calcitonin receptor, which had not been done previously due to difficulty in stabilizing the receptor–ligand interaction.

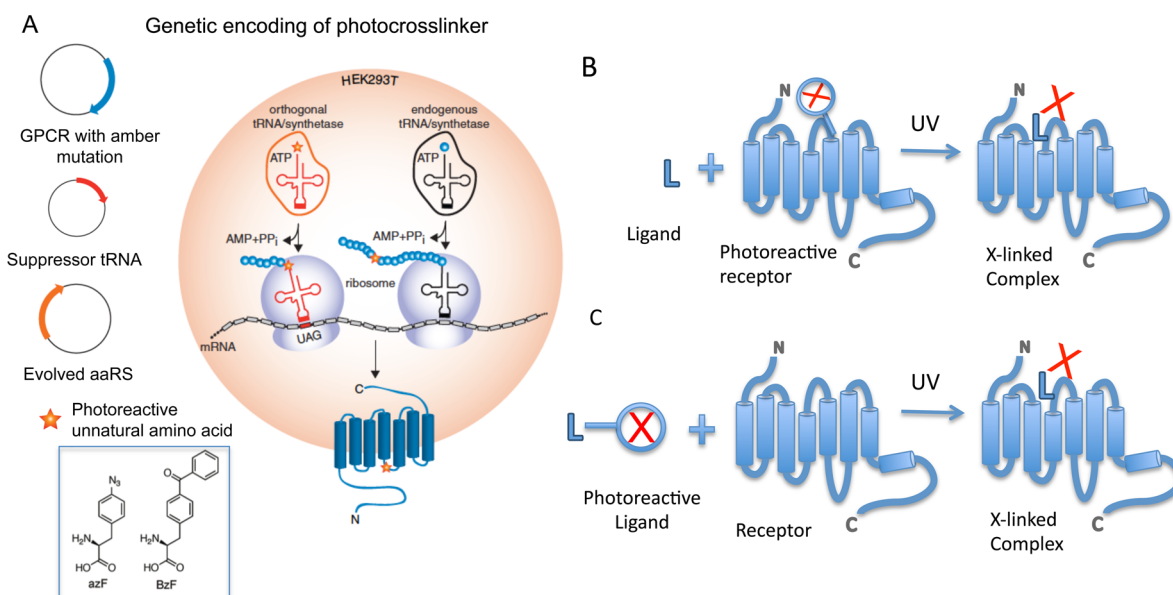
Hetero-bifunctional reagents are perhaps the most common and effective tools for cross-linking peptide ligands to their cognate receptors. These types of reagents can derivatize peptide ligands prior to receptor binding or once the ligand–receptor complex is formed. The prototypical structure of hetero-bifunctional reagents commonly includes a chemical cross-linking group attached via a linker to a photoactivatable cross-linking group. Here we describe several examples of hetero-bifunctional molecules that have been used to study GPCR–ligand interactions.

Labeling of primary amino groups in peptide ligands, either at the free amino group at the N-terminus or at lysine residues, can be carried out in several different ways. *N*-Hydroxysuccinimide ester or succinimidyl-containing molecules react with all accessible primary amines. The N-terminus of oxytocin was labeled specifically using the *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoylglycine because no lysines are present in this peptide.<sup>25</sup> Other cases, in which more than one primary amine is present, require protecting groups to label only one position. The parathyroid hormone was labeled specifically on a lysine with succinimidyl *p*-(3-iodobenzoyl)benzoate by using an Fmoc-protecting group on the N-terminal residue.<sup>26</sup> One reagent that specifically labels the  $\epsilon$ -amino group of lysines without reacting with the free amino group at the N-terminus is 4-fluoro-3-nitrophenylazide. This reagent was shown to label a lysine in glucagon, and the location of the photoactivatable group was characterized by Edman degradation.<sup>27,28</sup>

Another common functional group found in peptides is a carboxyl group, but derivatizing peptides at carboxyls is uncommon due to the high abundance of aspartic and glutamic acid residues in most peptides. In general, the greater the number of positions in a peptide that are labeled, the more difficult it is to purify a homogeneous analogue and the more likely it is that its binding to the receptor will be perturbed. One reagent used for carboxyl group modification is *N*-(5-azido-2-nitrophenyl)-2-ethylenediamine. In one report, this reagent labeled the carboxyl groups on  $\alpha$ -thrombin via a carbodiimide condensation reaction. The photoreactive  $\alpha$ -thrombin derivative was not found to have significantly altered binding to cell surface receptors. In addition, this ligand was found to cross-link specifically to a ~50-kDa receptor on mouse embryo cells.<sup>29</sup> Alternatively, peptide ligands can also be derivatized on tryptophan residues, which can be specifically labeled with sulfonyl chloride reagents that react with the side chain indole ring. Glucagon and corticotropin are two examples of peptide hormones that have been labeled with 2-nitro-5-azidophenyl-sulfonyl chloride for photoaffinity studies.<sup>30,31</sup>

Hetero-bifunctional reagents can also be added to a receptor–ligand complex after the complex is formed. In this scenario cross-linking should occur between the ligand and receptor of interest. However, since these types of reagents





**Figure 2.** Strategies to facilitate photochemical cross-linking of GPCR–ligand complexes. (A) Photoactivatable cross-linkers can be incorporated into GPCRs expressed in mammalian cells using amber codon suppression technology. In principle, any amino acid residue in the receptor can be substituted by a UAA, typically azF or BzF. Cells must be cotransfected with the receptor gene contained an amber codon mutation along with the appropriate orthogonal amino acyl-tRNA synthetase (aaRS) and suppressor tRNA genes. Media is supplemented with the desired UAA. (B) Using a receptor with a genetically encoded photoreactive amino acid allows UV-induced cross-linking to unmodified ligands directly in cells. One advantage of this approach is that many sites can be tested in parallel, and if cross-linking occurs, the site of the cross-link on the receptor is known. (C) Photoreactive ligands prepared as outlined in Figure 1 can also be cross-linked to expressed GPCRs.

tend to bind relatively nonspecifically, the results of positive cross-links can be complex and difficult to interpret. Despite the obvious drawbacks and low signal-to-noise problem inherent in attempts to cross-link with nonspecific reagents, the procedure can be relatively straightforward since tedious chemical synthesis of photoactivatable ligands is not formally needed. Another advantage of this approach is that native complexes between ligand and receptor are formed before the cross-linking reaction, and neither the ligand nor the receptor is altered before photolysis. This strategy was used to cross-link glucagon to the glucagon receptor in rat liver membranes. The hetero-bifunctional cross-linking reagent hydroxysuccinimide-*p*-azidobenzoate was added to membranes that were preincubated with  $^{125}$ I-glucagon. Photolysis resulted in the covalent cross-link of the labeled glucagon to a 53-kDa protein.<sup>32</sup>

**Introducing Photoreactive Side Chains into Peptide Ligands.** Photoreactive molecules can also be introduced into peptide ligands during peptide synthesis through the incorporation of amino acid derivatives or UAAs. This strategy enables the incorporation of the photo-cross-linker at any position in the peptide, whereas derivatization postsynthesis depends on the location of an available functional group. Benzophenone has been introduced into GPCR ligands in both of these manners. One report derivatized a lysine precursor prior to peptide synthesis with *p*-benzoylbenzoic acid.<sup>33</sup> The benzophenone-labeled lysine was introduced into parathyroid hormone and calcitonin during peptide synthesis.<sup>34,35</sup> Kauer et al. synthesized another benzophenone-containing UAA, *p*-benzoyl-L-phenylalanine (BzF).<sup>36,37</sup> BzF has subsequently been used in the development of many GPCR-targeted photoaffinity ligands. BzF scanning can be carried out where BzF is incorporated successively at each position of a peptide ligand analogue. In one notable example, BzF scanning was carried out

on substance P, which led to the identification of two specific positions in which BzF in the peptide sequence resulted in analogues that cross-linked to the NK-1 receptor.<sup>38</sup> Additional analysis of the cross-linked complex using protease digestion and analysis of proteolytic products led to the identification of the location in the receptor in which the cross-link occurred.<sup>39,40</sup> The process of identifying the location of the cross-link in the receptor can be extremely involved and complicated, and in most cases precise identification of the site of a cross-link is not technically possible. Therefore, newer strategies described below have been devised to improve the likelihood of identifying sites of cross-linking; these strategies dramatically enhance the utility and sensitivity of cross-linking experiments.

The introduction of BzF into peptide ligands to create photoaffinity probes has been used commonly for the study of class B GPCRs, also known as the secretin- or glucagon-like receptors. The characteristic structure of class B GPCRs includes a medium-length N-terminal domain, also referred to as the ectodomain, which is important for binding peptide hormones. Extensive photo-cross-linking experiments have been performed to understand how a peptide hormone binds to its cognate ectodomain. For example, the binding of secretin analogues to the ectodomain of the secretin receptor has been studied in detail using cross-linking approaches.<sup>41</sup> In these studies BzF was incorporated at various positions in secretin, and the resulting  $^{125}$ I-labeled analogues were characterized and cross-linked to the receptor in native membranes. The general location of cross-linking in the receptor was identified through extensive digestion of the covalent receptor-analogue–ligand complex followed by detection of the  $^{125}$ I-labeled fragment in the resulting peptide mixture using sodium dodecyl sulfate polyacrylamide gel electrophoresis.<sup>42</sup> To improve the localization of the cross-link site, additional cyanogen bromide

cleavage sites were created in the receptor by using site-directed mutagenesis to introduce methionine residues.<sup>43,44</sup> A similar strategy was also used to study the binding interactions between the vasoactive intestinal peptide (VIP) and the human VPAC1 receptor.<sup>45–47</sup> A molecular model of the VIP–hVPAC1 complex was created using the NMR structure of VIP and the distance constraints identified from the cross-linking study.<sup>48</sup> This study is a notable demonstration of how photo-cross-linking data can be applied to create a more detailed model of a GPCR–ligand complex.

A UAA containing an aryl azide, *p*-azido-L-phenylalanine (azF), has also been synthesized.<sup>49</sup> Since this amino acid is unstable under the conditions required for peptide synthesis,<sup>50</sup> another method was needed to incorporate azF into peptides. A precursor ion, L-4-nitrophenylalanine, was incorporated at the position of interest during synthesis, and then the nitro group was converted to an azido group through additional chemical reactions.<sup>51</sup> This method has been used to create photoaffinity analogues of angiotensin II and kinins.<sup>52,53</sup>

More recently derivatives of leucine and methionine have been generated to contain a diazirine group. These amino acids are referred to as photoleucine and photomethionine, respectively,<sup>54</sup> and were originally created for the incorporation of photoreactive groups into proteins expressed in cells since they can be recognized by the endogenous transfer RNA (tRNA) synthetases for leucine and methionine. Photoleucine has been incorporated into the novel GPCR lipopeptide ligands pepducins to investigate their mechanism of action.<sup>55</sup> This photo-cross-linking study demonstrated that a pepducin agonist of the C–X–C chemokine receptor 4 (CXCR4) cross-linked to the receptor, but the exact site of the cross-link was not identified. In the section that follows, we will discuss the methods available for the site-specific incorporation of photoactivatable cross-linkers into GPCRs to enable the identification or mapping of ligand binding sites.

## ■ GPCRS ENGINEERED TO CONTAIN PHOTOCROSS-LINKERS

A complementary technique to the use of photoaffinity ligands is to introduce photoactivatable groups at specific positions in a GPCR itself. An advantage to performing cross-linking experiments from the orientation of the receptor is that the exact site on the receptor of any potential covalent linkage is known in advance. Since the receptor is much larger than the ligand for any GPCR–ligand complex, and since the general aim of cross-linking experiments is to map the binding site of a ligand to its receptor, knowing the site of origin for a cross-link and the receptor can be a tremendous advantage. In general, as with the case of derivatizing the GPCR ligand, there are also two methods for introducing the photoreactive group into the receptor—either derivatize the receptor post-translationally or incorporate the unique functional group during protein translation (Figure 2). These methods both rely on heterologous overexpression of engineered receptors in culture using standard techniques of molecular biology as discussed below.

**Labeling Cysteines with Photocross-Linkers.** One way to introduce a photo-cross-linker into a GPCR is to use sulfhydryl chemistry and target the available cysteine residues after the receptor has been expressed and purified. Since all known GPCRs contain multiple cysteine residues, to attain specific labeling at only one site in the receptor requires that all other cysteine residues need to be removed by site-directed

mutagenesis. This technique was used to introduce an aryl azide at a specific cysteine in rhodopsin with the photo-cross-linking group *N*-((2-pyridyldithio)ethyl-4-azidosalicylamide (PEAS). In this report, the binding interactions between rhodopsin and the G protein, transducin, were investigated.<sup>56</sup> The receptor was first extracted from the cell membrane using detergent and bound to an immunoaffinity resin before being derivatized with the PEAS reagent. In principle this strategy could also be applied to investigate ligand–receptor interactions as long as the labeling reaction did not alter an essential conserved disulfide bond on the extracellular surface of the receptor. However, removal of all but one reactive cysteine residue, which can then be strategically situated as desired in the receptor sequence, is a painstaking process that can take years to accomplish. Many GPCRs have at least one cysteine residue that is essential for the correct formation of a receptor's tertiary structure.<sup>57,58</sup> In addition, many ligand binding events that lead to a ternary complex between receptor and G protein require the receptor to be in a native-like membrane environment. Incorporating the photoreactive group into a receptor during protein translation overcomes this limitation as discussed in the next section.

**UAA Mutagenesis.** The strategy of using amber stop codon suppression to incorporate amino acids with unique functional groups into proteins expressed in cells was developed most notably by Schultz and co-workers.<sup>59</sup> This methodology involves the engineering of orthogonal suppressor tRNA and amino acyl tRNA pairs and was originally advanced primarily for use in *E. coli* expression systems. For photo-cross-linking studies, the tRNA synthetases were developed for the two relevant UAAs, BzF and azF.<sup>60,61</sup> Since these reports, the amber codon suppression technique has been adapted and enhanced to incorporate BzF and azF into GPCRs expressed in yeast<sup>62,63</sup> and mammalian cells.<sup>64</sup>

The prototypical yeast GPCR, Ste2p, was the first yeast receptor reported to have a UAA incorporated at a specific site in the receptor. In this study BzF was introduced into Ste2p expressed in *Saccharomyces cerevisiae*.<sup>62</sup> The Ste2p BzF mutants were then examined for cross-linking to the Ste2p peptide ligand,  $\alpha$  factor. Several positions in the receptor were reported to result in covalent cross-links to biotinylated  $\alpha$  factor.<sup>62</sup> This was the first example of a covalent cross-link between an engineered GPCR with UAAs and a ligand.

The introduction of UAAs into GPCRs expressed in mammalian cells was first demonstrated using rhodopsin and C–C chemokine receptor 5 (CCR5). Heterologous expression of rhodopsin and CCR5 UAA mutants in human embryonic kidney (HEK) 293T cells resulted in the expression of functional receptors at the cell surface.<sup>64,65</sup> The application of BzF and azF to identify a ligand binding interface on a GPCR was validated using CXCR4 and the CXCR4-specific inhibitor T140. The data from this targeted photo-cross-linking experiment were evaluated using the crystal structure of CXCR4 bound to a homologous T140 peptide, CVX15.<sup>66,67</sup> A comparison of the cross-linking data to the crystal structure led to an estimation of the distance dependence of the BzF cross-linking reaction, which suggested that the photogenerated carbene in BzF needs to be within 3 Å of its target to form a covalent bond. This observation is in agreement with other reports on the distance requirement for benzophenone-based cross-linking.<sup>68</sup>

The BzF- and azF-targeted photo-cross-linking strategy has also been applied to identify ligand binding sites on the

corticotropin-releasing factor receptor<sup>68</sup> and CCR5.<sup>69</sup> In the CCR5 study, the binding site of the FDA-approved small molecule drug maraviroc was investigated. CCR5 is a human immunodeficiency virus type 1 (HIV-1) coreceptor required for cellular entry, and maraviroc is the first patient-approved HIV-1 cellular entry inhibitor that targets a GPCR. This report demonstrates that photoactivatable UAAs can be used to investigate the binding site of therapeutic drugs without modification to the drug's structure and could be useful in the design and development of future GPCR-based therapeutics.

## ■ INSIGHTS INTO THE STRUCTURE AND FUNCTION OF GPCR-LIGAND COMPLEXES

Over the past five years there has been a significant increase in the number of solved GPCR crystal structures, and the rate of newly available structures is increasing dramatically as enabling technologies have become disseminated. These structures provide atomic-level information about one static structure of a receptor, usually in complex with a ligand or drug. The conditions required for standard crystallographic techniques are not always amenable for the analysis of every receptor–ligand complex. Photoactivatable cross-linking studies are one alternative approach to gain structural information about these complexes. The distance dependency of these photolytic reactions allows for the precise mapping of biological interactions. In fact, targeted photo-cross-linking and crystallography are complementary methods because the systematic interpretation of cross-linking data requires knowledge of structure. The availability of just a single structure of a GPCR in complex with a ligand can facilitate cell-based photo-cross-linking studies with large numbers of ligands or ligand analogues. The only limiting factor is the problem of detecting a cross-link in cases where appropriately labeled ligands are not readily available.

Since the origins of photo-cross-linking 50 years ago, many studies have been performed to investigate the interactions between GPCRs and their ligands. This review only covers a small portion of the many noteworthy papers. However, the recent coincident advances in the structural biology of GPCRs and the UAA mutagenesis of GPCRs have led to a resurgence of interest in targeted photo-cross-linking to probe receptor–ligand binding sites. By interpreting targeted cross-linking data in light of available crystal structures, more accurate models of receptor–ligand complexes can be created. The complementarity of these two structural techniques has the potential to advance our understanding of the structure and function of GPCR–ligand interactions.

## ■ AUTHOR INFORMATION

### Corresponding Author

\*E-mail: Thomas.Sakmar@rockefeller.edu.

### Notes

The authors declare no competing financial interest.

## ■ REFERENCES

- (1) Singh, A., Thornton, E. R., and Westheimer, F. H. (1962) The photolysis of diazoacetylchymotrypsin. *J. Biol. Chem.* 237, 3006–3008.
- (2) Chowdhry, V., and Westheimer, F. H. (1979) Photoaffinity labeling of biological systems. *Annu. Rev. Biochem.* 48, 293–325.
- (3) Fleet, G. W. J., Porter, R. R., and Knowles, J. R. (1969) Photoaffinity labelling of antibodies with aryl nitrene as reactive group. *Nature* 224, 511–512.
- (4) Nakayama, T. A., and Khorana, H. G. (1990) Synthesis of a new photoactivatable analogue of 11-*cis*-retinal. *J. Org. Chem.* 1990 (55), 4953–4956.
- (5) Nakayama, T. A., and Khorana, H. G. (1990) Orientation of retinal in bovine rhodopsin determined by cross-linking using a photoactivatable analog of 11-*cis*-retinal. *J. Biol. Chem.* 265 (26), 15762–15769.
- (6) Galardy, R. E., Craig, L. C., Jamieson, J. D., and Printz, M. P. (1974) Photoaffinity labeling of peptide hormone binding sites. *J. Biol. Chem.* 249 (11), 3510–3518.
- (7) Bayley, H., and Knowles, J. R. (1978) Photogenerated reagents for membrane labeling. 2. Phenylcarbene and adamantylidene formed within the lipid bilayer. *Biochemistry* 17 (12), 2420–2423.
- (8) Dorman, G., and Prestwich, G. D. (1994) Benzophenone photophores in biochemistry. *Biochemistry* 33 (19), 5661–5673.
- (9) Gritsan, N. P., Gudmundsdottir, A. D., Tigelaar, D., Zhu, Z., Karney, W. L., Hadad, C. M., and Platz, M. S. (2001) A laser flash photolysis and quantum chemical study of the fluorinated derivatives of singlet phenylnitrene. *J. Am. Chem. Soc.* 123 (9), 1951–1962.
- (10) Ganjian, I., Pettei, M. J., Nakanishi, K., and Kaissling, K. (1978) A photoaffinity-labelled insect sex pheromone for the moth *Antheraea polyphemus*. *Nature* 271, 157–158.
- (11) Vogt, R. G., Prestwich, G. D., and Riddiford, L. M. (1988) Sex pheromone receptor proteins. Visualization using a radiolabeled photoaffinity analog. *J. Biol. Chem.* 263 (8), 3952–3959.
- (12) Overington, J. P., Al-Lazikani, B., and Hopkins, A. L. (2006) How many drug targets are there? *Nat. Rev. Drug Discovery* 5, 993–996.
- (13) Nishikori, K., Noshiro, O., Sano, K., and Maeno, H. (1980) Characterization, solubilization, and separation of two distinct dopamine receptors in canine caudate nucleus. *J. Biol. Chem.* 255 (22), 10909–10915.
- (14) Kuno, T., and Tanaka, C. (1981) Identification of the D-1 dopamine receptor subunit in rat striatum after photoaffinity labeling. *Brain Res.* 230 (1–2), 417–421.
- (15) Takayanagi, I., Yoshioka, M., Takagu, K., and Tamura, Z. (1976) Photoaffinity labeling of the beta-adrenergic receptors and receptor reserve for isoprenaline. *Eur. J. Pharmacol.* 35 (1), 121–125.
- (16) Darfler, F. J., and Marinetti, G. V. (1977) Synthesis of a photoaffinity probe for the beta-adrenergic receptor. *Biochem. Biophys. Res. Commun.* 79 (1), 1–7.
- (17) Wrenn, S. M., Jr., and Homcy, C. J. (1980) Photoaffinity label for the beta-adrenergic receptor: synthesis and effects on isoproterenol-stimulated adenylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.* 77 (8), 4449–4453.
- (18) Rashidbaigi, A., and Ruoho, A. E. (1981) Iodoazidobenzylpindolol, a photoaffinity probe for the beta-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.* 78 (3), 1609–1613.
- (19) Lavin, T. N., Heald, S. L., Jeffs, P. W., Shorr, R. G., Lefkowitz, R. J., and Caron, M. G. (1981) Photoaffinity labeling of the beta-adrenergic receptor. *J. Biol. Chem.* 256 (22), 11944–11950.
- (20) Stadel, J. M., Nambi, P., Lavin, T. N., Heald, S. L., Caron, M. G., and Lefkowitz, R. J. (1982) Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase. Structural alterations in the beta-adrenergic receptor revealed by photoaffinity labeling. *J. Biol. Chem.* 257 (16), 9242–9245.
- (21) Burgermeister, W., Hekman, M., and Helmreich, E. J. (1982) Photoaffinity labeling of the beta-adrenergic receptor with azide derivatives of iodoacetyl pindolol. *J. Biol. Chem.* 257 (9), 5306–5311.
- (22) Burgermeister, W., Nassal, M., Wieland, T., and Helmreich, E. J. (1983) A carbene-generating photoaffinity probe for beta-adrenergic receptors. *Biochim. Biophys. Acta* 729 (2), 219–228.
- (23) Gorman, J. J., and Folk, J. E. (1980) Transglutaminase amine substrates for photochemical labeling and cleavable cross-linking of proteins. *J. Biol. Chem.* 255 (3), 1175–1180.
- (24) Moseley, J. M., Findlay, D. M., Martin, T. J., and Gorman, J. J. (1982) Covalent cross-linking of a photoactive derivative of calcitonin to human breast cancer cell receptors. *J. Biol. Chem.* 257 (10), 5846–5851.



- (25) Stadel, J. M., Goodman, D. B., Galaray, R. E., and Rasmussen, H. (1978) Synthesis and characterization of 2-nitro-5-aziodobenzoyl-glycyloxytocin, an oxytocin photoaffinity label. *Biochemistry* 17 (8), 1403–1408.
- (26) Zhou, A. T., Bessalle, R., Bisello, A., Nakamoto, C., Rosenblatt, M., Suva, L. J., and Chorev, M. (1997) Direct mapping of an agonist-binding domain within the parathyroid hormone/parathyroid hormone-related protein receptor by photoaffinity crosslinking. *Proc. Natl. Acad. Sci. U.S.A.* 94 (8), 3644–3649.
- (27) Bregman, M. D., and Levy, D. (1977) Labeling of glucagon binding components in hepatocyte plasma membranes. *Biochem. Biophys. Res. Commun.* 78 (2), 584–590.
- (28) Bregman, M. D., Cheng, S., and Levy, D. (1978) Synthesis and properties of a photoaffinity probe for the glucagon receptor in hepatocyte plasma membranes. *Biochim. Biophys. Acta* 539 (4), 489–495.
- (29) Carney, D. H., Glenn, K. C., Cunningham, D. D., Das, M., Fox, C. F., and Fenton, J. W., 2nd (1979) Photoaffinity labeling of a single receptor for  $\alpha$ -thrombin on mouse embryo cells. *J. Biol. Chem.* 254 (14), 6244–6247.
- (30) Ramachandran, J., Muramoto, K., Kenez-Keri, M., Keri, G., and Buckley, D. I. (1980) Photoaffinity labeling of corticotropin receptors. *Proc. Natl. Acad. Sci. U.S.A.* 77 (7), 3967–3970.
- (31) Demoliou, C. D., and Epan, R. M. (1980) Synthesis and characterization of a hetero-bifunctional photoaffinity reagent for modification of tryptophan residues and its application to the preparation of a photoreactive glucagon derivative. *Biochemistry* 19 (20), 4539–4546.
- (32) Johnson, G. L., MacAndrew, V. I., Jr., and Pilch, P. F. (1981) Identification of the glucagon receptor in rat liver membranes by photoaffinity crosslinking. *Proc. Natl. Acad. Sci. U.S.A.* 78 (2), 875–878.
- (33) Gorka, J., McCourt, D. W., and Schwartz, B. D. (1989) Automated synthesis of a C-terminal photoprobe using combined Fmoc and t-Boc synthesis strategies on a single automated peptide synthesizer. *Pept. Res.* 2 (6), 376–380.
- (34) Nakamoto, C., Behar, V., Chin, K. R., Adams, A. E., Suva, L. J., Rosenblatt, M., and Chorev, M. (1995) Probing the bimolecular interactions of parathyroid hormone with the human parathyroid hormone/parathyroid hormone-related protein receptor. 1. Design, synthesis, and characterization of photoreactive benzophenone-containing analogs of parathyroid hormone. *Biochemistry* 34 (33), 10546–10552.
- (35) Suva, L. J., Flannery, M. S., Caulfield, M. P., Findlay, D. M., Juppner, H., Goldring, S. R., Rosenblatt, M., and Chorev, M. (1997) Design, synthesis and utility of novel benzophenone-containing calcitonin analogs for photoaffinity labeling the calcitonin receptor. *J. Pharmacol. Exp. Ther.* 283 (2), 876–884.
- (36) Kauer, J. C., Erickson-Viitanen, S., Wolfe, H. R., Jr., and DeGrado, W. F. (1986) *p*-Benzoyl-L-phenylalanine, a new photo-reactive amino acid. Photolabeling of calmodulin with a synthetic calmodulin-binding peptide. *J. Biol. Chem.* 261 (23), 10695–10700.
- (37) Herblin, W. F., Kauer, J. C., and Tam, S. W. (1987) Photoinactivation of the  $\mu$  opioid receptor using a novel synthetic morphiceptin analogue. *Eur. J. Pharmacol.* 139 (3), 273–279.
- (38) Li, Y. M., Marnerakis, M., Stimson, E. R., and Maggio, J. E. (1995) Mapping peptide-binding domains of the substance P (NK-1) receptor from P388D1 cells with photolabile agonists. *J. Biol. Chem.* 270 (3), 1213–1220.
- (39) Kage, R., Leeman, S. E., Krause, J. E., Costello, C. E., and Boyd, N. D. (1996) Identification of methionine as the site of covalent attachment of a *p*-benzoyl-phenylalanine-containing analogue of substance P on the substance P (NK-1) receptor. *J. Biol. Chem.* 271 (42), 25797–25800.
- (40) Boyd, N. D., Kage, R., Dumas, J. J., Krause, J. E., and Leeman, S. E. (1996) The peptide binding site of the substance P (NK-1) receptor localized by a photoreactive analogue of substance P: presence of a disulfide bond. *Proc. Natl. Acad. Sci. U.S.A.* 93 (1), 433–437.
- (41) Dong, M., Wang, Y., Pinon, D. I., Hadac, E. M., and Miller, L. J. (1999) Demonstration of a direct interaction between residue 22 in the carboxyl-terminal half of secretin and the amino-terminal tail of the secretin receptor using photoaffinity labeling. *J. Biol. Chem.* 274 (2), 903–909.
- (42) Dong, M., Wang, Y., Hadac, E. M., Pinon, D. I., Holicky, E., and Miller, L. J. (1999) Identification of an interaction between residue 6 of the natural peptide ligand and a distinct residue within the amino-terminal tail of the secretin receptor. *J. Biol. Chem.* 274 (27), 19161–19167.
- (43) Dong, M., Asmann, Y. W., Zang, M., Pinon, D. I., and Miller, L. J. (2000) Identification of two pairs of spatially approximated residues within the carboxyl terminus of secretin and its receptor. *J. Biol. Chem.* 275 (34), 26032–26039.
- (44) Zang, M., Dong, M., Pinon, D. I., Ding, X. Q., Hadac, E. M., Li, Z., Lybrand, T. P., and Miller, L. J. (2003) Spatial approximation between a photolabile residue in position 13 of secretin and the amino terminus of the secretin receptor. *Mol. Pharmacol.* 63 (5), 993–1001.
- (45) Tan, Y. V., Couvineau, A., Van Rampelbergh, J., and Laburthe, M. (2003) Photoaffinity labeling demonstrates physical contact between vasoactive intestinal peptide and the N-terminal ectodomain of the human VPAC1 receptor. *J. Biol. Chem.* 278 (38), 36531–36536.
- (46) Tan, Y. V., Couvineau, A., and Laburthe, M. (2004) Diffuse pharmacophoric domains of vasoactive intestinal peptide (VIP) and further insights into the interaction of VIP with the N-terminal ectodomain of human VPAC1 receptor by photoaffinity labeling with [Bpa6]-VIP. *J. Biol. Chem.* 279 (37), 38889–38894.
- (47) Tan, Y. V., Couvineau, A., Murail, S., Ceraudo, E., Neumann, J. M., Lacapere, J. J., and Laburthe, M. (2006) Peptide agonist docking in the N-terminal ectodomain of a class II G protein-coupled receptor, the VPAC1 receptor. Photoaffinity, NMR, and molecular modeling. *J. Biol. Chem.* 281 (18), 12792–12798.
- (48) Ceraudo, E., Murail, S., Tan, Y. V., Lacapere, J. J., Neumann, J. M., Couvineau, A., and Laburthe, M. (2008) The vasoactive intestinal peptide (VIP)  $\alpha$ -helix up to C-terminus interacts with the N-terminal ectodomain of the human VIP/pituitary adenylate cyclase-activating peptide 1 receptor: photoaffinity, molecular modeling, and dynamics. *Mol. Endocrinol.* 22 (1), 147–155.
- (49) Schwyzler, R., and Caviezel, M. (1971) *p*-Azido-L-phenylalanine: a photo-affinity “probe” related to tyrosine. *Helv. Chim. Acta* 54 (5), 1395–1400.
- (50) Escher, E. (1977) Isolation and structure analysis of a photoproduct of the new photoaffinity label *p*-nitrophenylalanine. *Helv. Chim. Acta* 60 (2), 339–341.
- (51) Escher, E. H., Nguyen, T. M., Robert, H., St-Pierre, S. A., and Regoli, D. C. (1978) Photoaffinity labeling of the angiotensin II receptor. 1. Synthesis and biological activities of the labeling peptides. *J. Med. Chem.* 21 (9), 860–864.
- (52) Escher, E. H., and Guillemette, G. (1979) Photoaffinity labeling of the angiotensin II receptor. 3. Receptor inactivation with photolabile hormone analogues. *J. Med. Chem.* 22 (9), 1047–1050.
- (53) Escher, E., Laczko, E., Guillemette, G., and Regoli, D. (1981) Biological activities of photoaffinity labeling analogues of kinins and their irreversible effects on kinin receptors. *J. Med. Chem.* 24 (12), 1409–1413.
- (54) Suchanek, M., Radzikowska, A., and Thiele, C. (2005) Photoleucine and photomethionine allow identification of protein-protein interactions in living cells. *Nat. Methods* 2 (4), 261–267.
- (55) Janz, J. M., Ren, Y., Looby, R., Kazmi, M. A., Sachdev, P., Grunbeck, A., Haggis, L., Chinnapen, D., Lin, A. Y., Seibert, C., McMurtry, T., Carlson, K. E., Muir, T. W., Hunt, S., 3rd, and Sakmar, T. P. (2011) Direct interaction between an allosteric agonist pepducin and the chemokine receptor CXCR4. *J. Am. Chem. Soc.* 133 (40), 15878–15881.
- (56) Cai, K., Itoh, Y., and Khorana, F. C. (2001) Mapping of contact sites in complex formation between transducin and light-activated rhodopsin by covalent crosslinking: Use of a photoactivatable reagent. *Proc. Natl. Acad. Sci. U.S.A.* 98 (9), 4877–4882.



- (57) Karnik, S. S., Sakmar, T. P., Chen, H. B., and Khorana, H. G. (1988) Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* 85 (22), 8459–8463.
- (58) Ward, S. D., Hamdan, F. F., Bloodworth, L. M., and Wess, J. (2002) Conformational changes that occur during M3 muscarinic acetylcholine receptor activation probed by the use of an in situ disulfide cross-linking strategy. *J. Biol. Chem.* 277 (3), 2247–2257.
- (59) Anthony-Cahill, S. J., Griffith, M. C., Noren, C. J., Suich, D. J., and Schultz, P. G. (1989) Site-specific mutagenesis with unnatural amino acids. *Trends Biochem. Sci.* 14 (10), 400–403.
- (60) Chin, J. W., Martin, A. B., King, D. S., Wang, L., and Schultz, P. G. (2002) Addition of a photocrosslinking amino acid to the genetic code of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 99 (17), 11020–11024.
- (61) Chin, J. W., Santoro, S. W., Martin, A. B., King, D. S., Wang, L., and Schultz, P. G. (2002) Addition of *p*-azido-L-phenylalanine to the genetic code of *Escherichia coli*. *J. Am. Chem. Soc.* 124 (31), 9026–9027.
- (62) Huang, L. Y., Umanah, G., Hauser, M., Son, C., Arshava, B., Naider, F., and Becker, J. M. (2008) Unnatural amino acid replacement in a yeast G protein-coupled receptor in its native environment. *Biochemistry* 47 (20), 5638–5648.
- (63) Umanah, G., Huang, L. Y., Schultz, P. G., Naider, F., and Becker, J. M. (2009) Incorporation of the unnatural amino acid *p*-benzoyl-L-phenylalanine (Bpa) into a G protein-coupled receptor in its native context. *Adv. Exp. Med. Biol.* 611, 333–335.
- (64) Ye, S. X., Kohrer, C., Huber, T., Kazmi, M., Sachdev, P., Yan, E. C. Y., Bhagat, A., RajBhandary, U. L., and Sakmar, T. P. (2008) Site-specific incorporation of keto amino acids into functional G protein-coupled receptors using unnatural amino acid mutagenesis. *J. Biol. Chem.* 283 (3), 1525–1533.
- (65) Ye, S. X., Huber, T., Vogel, R., and Sakmar, T. P. (2009) FTIR analysis of GPCR activation using azido probes. *Nat. Chem. Biol.* 5 (6), 397–399.
- (66) Grunbeck, A., Huber, T., Sachdev, P., and Sakmar, T. P. (2011) Mapping the ligand-binding site on a G protein-coupled receptor (GPCR) using genetically encoded photo-cross-linkers. *Biochemistry* 50 (17), 3411–3413.
- (67) Wu, B., Chien, E. Y., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., Abagyan, R., Brooun, A., Wells, P., Bi, F. C., Hamel, D. J., Kuhn, P., Handel, T. M., Cherezov, V., and Stevens, R. C. (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science* 330 (6007), 1066–1071.
- (68) Coin, I., Perrin, M. H., Vale, W. W., and Wang, L. (2011) Photo-cross-linkers incorporated into G-protein-coupled receptors in mammalian cells: a ligand comparison. *Angew. Chem., Int. Ed.* 50 (35), 8077–81.
- (69) Grunbeck, A., Huber, T., Abrol, R., Trzaskowski, B., Goddard, W. A., and Sakmar, T. P. (2012) Genetically-encoded photo-cross-linkers map the binding site of an allosteric drug on a G protein-coupled receptor. *ACS Chem. Biol.* 7 (6), 967–972.