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Perspective

Biophysical approaches to G protein-coupled receptors: Structure, function and dynamics

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Summary

G protein-coupled receptors (GPCR) represent a large family of drug targets for which there is no high-resolution structural information. In order to understand the mechanisms of ligand recognition and receptor activation, there is a strong need for novel biophysical methods. In this Perspective we provide an overview of recent experimental approaches used to explore the molecular architecture and dynamics of GPCR and their interactions with ligands and G proteins using biophysical, non-crystallographic, methods.

Introduction

G protein-coupled receptors (GPCR) represent a large family of integral membrane proteins that transmit signals into cells in response to a variety of extracellular stimuli such as bioactive monoamines, peptide hormones of various sizes, chemokines, lipid analogues, amino acids and sensory stimuli. These receptors are targets of numerous important drugs and are also the targets of drug discovery programs in the pharmaceutical industry. In recent years, due to the application of molecular biology techniques, well over 100 GPCR have been identified, cloned and pharmacologically characterized. It is estimated that up to 1% of the human genome may be constituted of GPCR. Despite this explosion of information, the mechanisms of ligand recognition and of activation of these receptors remain unclear and there is a paucity of tertiary structure information.

Classical structural approaches are difficult for these membrane proteins and are hampered by the difficulties in obtaining and purifying large amounts of protein required for the production of crystals for high resolution X-ray or electron diffraction. Biochemical experiments, primary sequence analysis and mutagenesis data suggested that GPCR are formed of seven membrane spanning hydrophobic helices linked by extra- and intracellular loops of varying length. The N-terminal domain is extracellular, possibly N-glycosylated, whereas the C-terminal domain is intracellular and often palmitoylated on cysteine residues to create additional anchoring into the lipid bilayers. Indeed, direct determination of the projection structure of rhodopsin at low resolution supports the seven helices model and defines their topographic arrangement [1, 2].

Mutational mapping of binding sites revealed that GPCR exploit diverse strategies for ligand recognition, using either the transmembrane domain, the extracellular surface or even the N-terminal segment. G protein binding and activation involves cytoplasmic domains of GPCR. Unfortunately, to date there is no structural information on extra- or intracellular domains. One important feature that is currently emerging is the dynamic nature of GPCR. A number of different conformations may exist and interchange which can selectively interact with agonist or antagonist ligands and activate G proteins with important functional consequences. Because detailed structural information is important to the understanding of the

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mechanisms of ligand recognition and activation of G protein-coupled receptors, as well as in the design of therapeutic agents, there is a need to develop novel biochemical and biophysical methods. The aim of this Perspective is to review and discuss opportunities and limitations of the particular biophysical methods to investigate the structure and dynamics of GPCR with special emphasis on the fluorescence-based methods recently developed in our laboratory. Crystallographic or biochemical methods are not included.

Structural information from biophysical studies

Some of the key structural questions that biophysical approaches can address relate to the molecular architecture of GPCR including intramolecular distances between specific sites in the receptor protein and also intermolecular distances between receptors and interacting partners such as ligands, G proteins or lipids. Also it is important to define water-membrane boundaries and exposed loop regions, the length of alpha helical segments and the orientation of these helices. The use of time-resolved techniques has the potential to monitor conformational changes occurring during the functional activation of GPCR and to understand the molecular basis for receptor function.

Labeling of GPCR with biophysical probes

A common prerequisite to biophysical studies of GPCR is the introduction of extrinsic probes such as spin labels, fluorophores or isotopically labeled amino acids in the receptor protein. This is a real challenge because, owing to their relatively large size of more than 300 amino acids, GPCR cannot be chemically synthesized de novo. Therefore, two approaches have been followed for the introduction of reporter groups in GPCR: (1) chemical modification of uniquely reactive amino acid side chains (cysteinyl and lysyl) which can serve as attachment points for labels, and (2) biosynthetic incorporation of unnatural amino acids by nonsense codon suppression.

The β2 adrenergic receptor has been labeled on native cysteines with the fluorescent group nitrobenzoxadiazole (NBD) (Figure 1) using a sulfhydryl-specific NBD analogue which does not perturb the pharmacological properties of the receptor [3]. One problem with this experimental method is the lack of selectivity resulting in the uncontrolled labeling at multiple sites in the receptor. This can be avoided by using singly reactive sites. To label rhodopsin with spin markers, the groups of Khorana and Hubbell have used chemical modification of single cysteine substitution mutants with a sulfhydryl-specific nitroxide reagent (Figure 1) [4, 5]. Cysteine mutants were expressed in COS cells and purified by immunoaffinity and reconstituted in membranes with the 11-*cis*-retinal chromophore. Any chemical modification approach requires a robust expression, purification and reconstitution system because the receptor protein must be isolated prior to labeling.

Biosynthetic incorporation of biophysical probes

In our laboratory, we have applied the technique of nonsense suppression mutagenesis to the site-specific labeling of GPCR. In this recently developed methodology, a nonsense stop codon (for instance UAG, amber) is created in the receptor coding sequence, at the site selected for unnatural mutagenesis, and is suppressed during translation by using an amber suppressor tRNA, chemically misacylated with the desired unnatural amino acid, to yield the mutant protein. This technique was originally developed for small soluble proteins [6–8] or bacteriorhodopsin [9] in an in vitro system capable of supporting protein biosynthesis, such as *E. coli* S30 extracts, rabbit reticulocyte lysate or wheat-germ extracts. When we attempted to express the tachykinin GPCR neurokinin-2 (NK2) receptor in an in vitro translation system, we found that the protein was made but the receptor was not active as assayed by ligand binding, thereby precluding the exploration of unnatural suppression mutagenesis techniques *in vitro* [10].

However, using heterologous expression in an intact cell system, the *Xenopus* oocyte, we introduced an unnatural fluorescent amino acid at known sites into the NK2 receptor by suppression of UAG nonsense codons with the aid of a specifically designed and chemically misacylated suppressor tRNA [11].

As shown in Figure 2 the key steps in this approach are: (1) the mutation of a selected site in NK2 cDNA to the TAG termination codon and the preparation of the cognate cRNA transcript containing the UAG codon; (2) the construction by chemical and enzymatic methods of a misacylated suppressor tRNA that recognizes the UAG stop codon and functions biosynthetically as a source of the unnatural amino acid; and (3) the heterologous expression of receptor

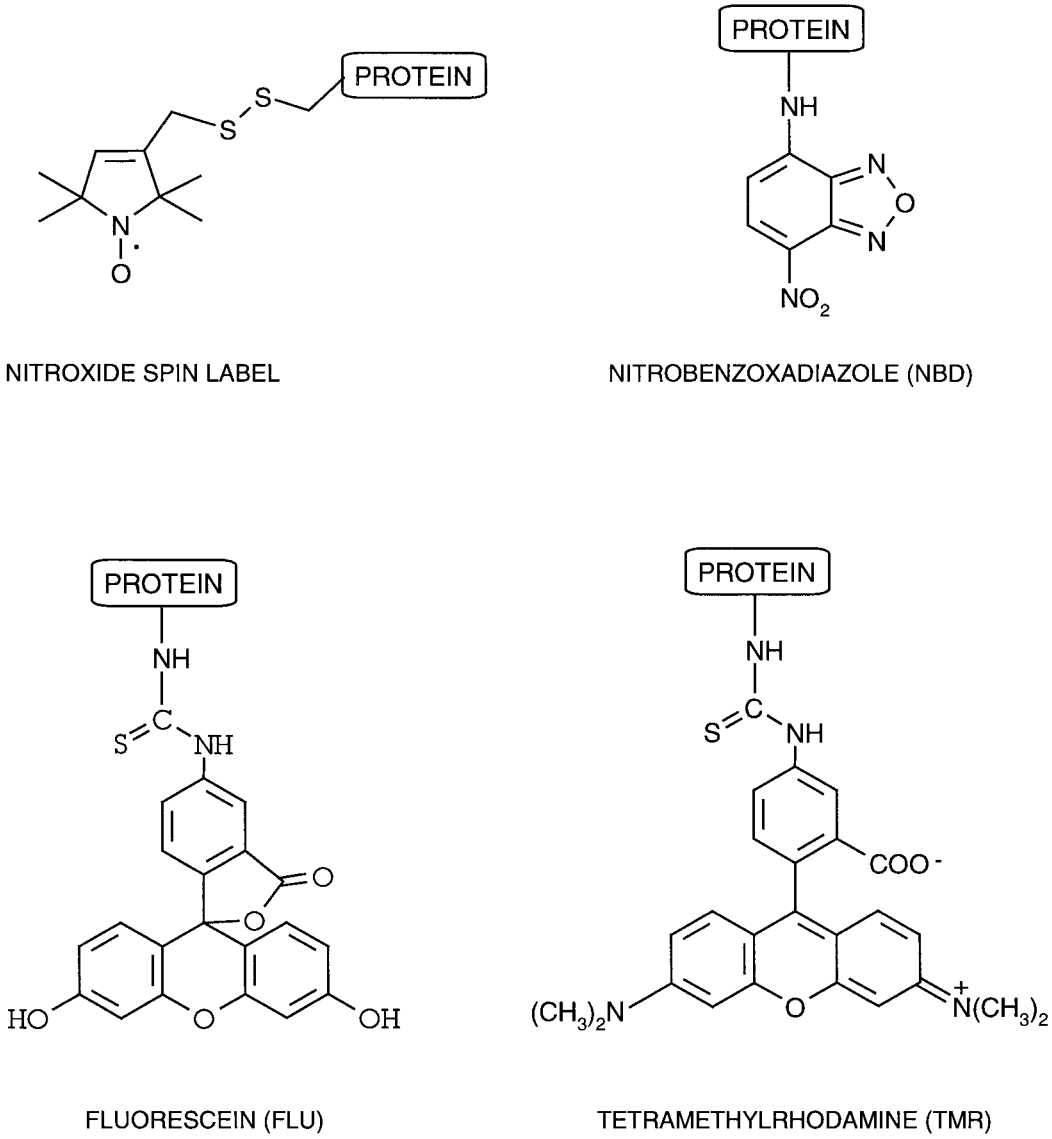


Figure 1. Chemical structures of representative fluorescent groups and spin labels used as biophysical probes.

containing unnatural amino acids in *Xenopus* oocytes. The oocyte cell translation machinery recognizes the exogenous aminoacylated suppressor tRNA and incorporates the fluorescent residue into the nascent protein at sites preselected by creation of a nonsense codon in the gene. To improve the efficiency of the incorporation, we designed and constructed a synthetic UAG suppressor tRNA (Sup tRNA-NBD, Figures 3 and 4) derived from yeast tRNA(Phe). This contained the anticodon CUA that recognizes the UAG stop mutation in the cRNA. The fluorescent amino acid NBD-Dap was acylated chemically to the 3′-end and two mutations (G20U and A73G) were introduced to abolish reacylation with natural amino acids by endogenous aminoacyl-tRNA synthetases. Functional activity of fluorescent NK2 receptor mutants in oocytes was characterized by Ca^{2+}-dependent inward chloride currents evoked by stimulation with neurokinin A. The K_d values for binding of a radiolabeled NK2 antagonist were comparable for the NBD mutants and wild-type NK2; i.e., in the nanomolar range. Numerous controls were made to insure the fidelity of suppression. The efficiency of suppression was evaluated by saturation binding of a radiolabeled antagonist to NK2 and

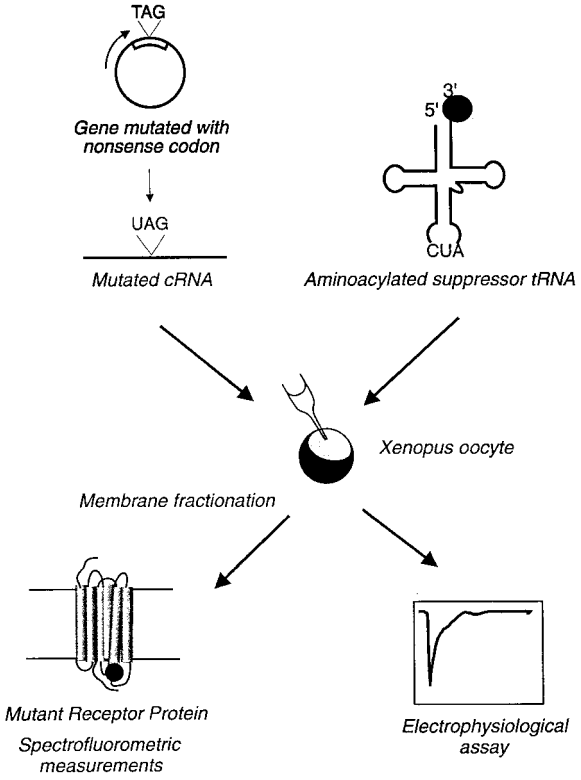


Figure 2. Strategy for fluorescent labeling of GPCR by biosynthetic incorporation of unnatural amino acids during heterologous expression in oocytes.

suppression mutants. The yields were in the 5–30% range.

In contrast to chemical modification methods, the unnatural suppression mutagenesis approach has allowed to produce singly labeled receptor proteins at a known site in a native membrane environment thus avoiding the purification and reconstitution steps. Also, native membranes contain more homogeneous populations of receptors with regard to functional coupling to signal transduction proteins.

Our data are the first report of the incorporation of a fluorescent unnatural amino acid into a membrane protein in intact cells by the method of nonsense codon suppression. Using this approach we produced more than 10 different site-specifically labeled fluorescent NK2 mutants in a native membrane environment [12]. We believe that this methodology can be generally applied to other integral membrane proteins such as receptors or channels.

Electron paramagnetic resonance (EPR) studies with spin labels

The structure and light activation of the GPCR rhodopsin, a membrane-bound photoreceptor protein of the vertebrate rod cell, have been investigated by EPR in a series of elegant experiments by the groups of Hubbell and Khorana. EPR directly detects the presence of free radicals (as for instance in nitroxide spin labels) and has the potential to reveal information on structure and motional dynamics at the labeled sites. The methodology has been developed and validated on the proton pump bacteriorhodopsin, an integral membrane protein thought to have the same overall topology as GPCR [13]. Recently, Hubbell, Khorana and co-workers applied the methodology to rhodopsin.

Topography and identification of regular secondary structure

An analysis of the EPR line shape of the attached nitroxide and its collision rate with polar and non-polar paramagnetic reagents in solution can give insights in the local structure as well as the location of the spin labeled residues relative to the hydrophobic/hydrophilic boundaries. Measurement of two collision parameters: one for a hydrophobic reagent, such as O_2, and the other for collision with a hydrophilic radical, such as the charged polar chromium(III)trioxalate (Crox) or neutrally polar reagents Ni(II)ethylenediamine diacetate (NiEDDA) and Ni(II)acetylacetonate (NiAA) are sufficient to resolve solvent exposed, lipid exposed and buried groups [14].

Nitroxide scanning mutagenesis through a sequence of regular secondary structure will in general reveal periodic variations in accessibility that can serve to identify the type of secondary structure and its orientation in the protein. This ‘nitroxide scanning’ experiment was illustrated by the series of spin-labeled bacteriorhodopsin mutants in the transmembrane E helix at positions 125–142 [15]. Oxygen accessibility for positions 131 to 138 varied with a periodicity of 3.6 residues, a suggesting an α-helical structure.

The topographical location of nitroxide labels attached to single cysteine mutants in the cytoplasmic domains of rhodopsin were investigated by EPR [4, 5]. Water-membrane interfaces were deduced from collisional quenching and were found compatible with predictions derived from the hydropathy analysis of the primary sequence.

In another study, the time-resolved EPR signal from spin-labeled rhodopsin in the native disc mem-

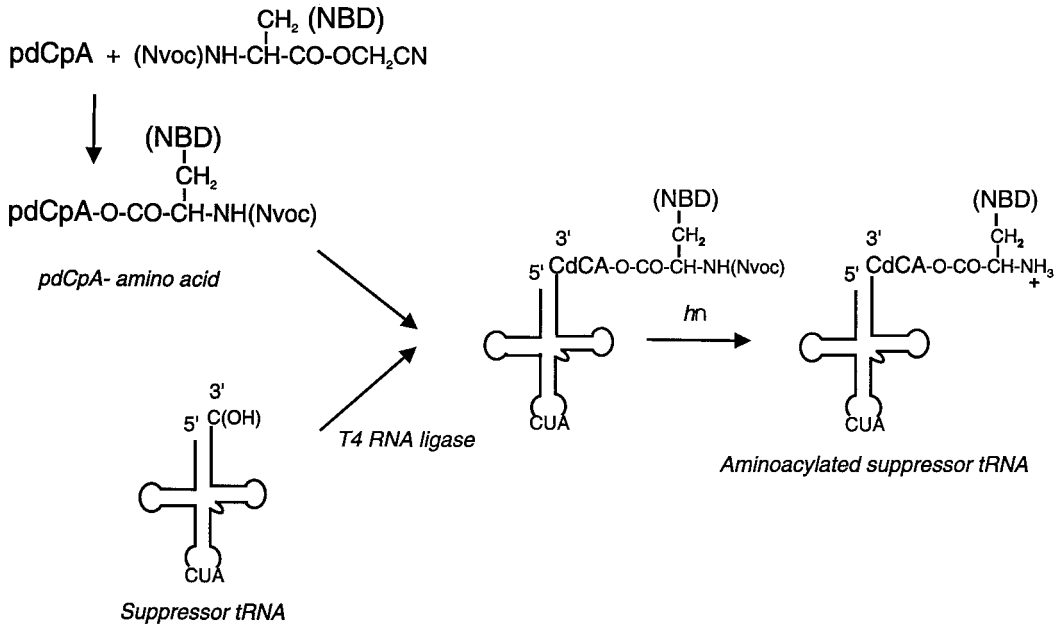


Figure 3. Scheme for the construction of a chemically misacylated suppressor tRNA.

brane revealed a light-triggered conformational transition in the millisecond range in the region of the second cytoplasmic loop coincident with the appearance of the active metarhodopsin II (MII) intermediate [16]. The changes were similar at three temperatures, indicating that the changes in tertiary interaction sensed by the label reflected receptor activation. The data are consistent with a small movement near the cytoplasmic end of the C helix which reverses upon formation of the MII state. This work provides an important link between the optical changes of the retinal chromophore and protein conformational states.

Recently another site-directed spin-labeling study of rhodopsin has been reported for the E-F interhelical loop, a transducin interaction site [17]. Accessibility and mobility data were used to determine the water-membrane boundaries and show that hydrophobic segments were α-helical. Moreover, periodicity of accessibility and mobilities of the spin-labeled side chains in the aqueous exposed E-F interhelical loop domain indicated that α-helices E and F extend beyond the lipid bilayer well into the loop. Nitroxide mobilities reflected differences in helix internal fluctuations, suggesting that the extension of helix E in the aqueous phase is more dynamic than that of helix F.

Tertiary interactions and conformational changes

Spin labels were also used to monitor local long-lived conformational changes associated with rhodopsin function. Rhodopsin undergoes a conformational change upon the isomerization of the 11-*cis*-retinal chromophore by light. This conformational change at the cytoplasmic surface leads to activation of transducin, the visual G protein.

Resek et al. [4] reported changes in tertiary interaction sensed by a spin label at position 140 in the C-D loop and at position 316 in the carboxy-terminal domain upon formation of the activated receptor state, the MII intermediate in the rhodopsin bleaching sequence. These loops are implicated in recognition of the G protein, transducin. Changes were not detected by spin labels in the A-B or E-F loops, indicating that the conformational change is not global. Light-dependent structural changes in the putative C-D interhelical loop of rhodopsin were also reported in a nitroxide scanning mutagenesis study (rhodopsin sequence 136–155) [5]. A possible interpretation of the results is that photoexcitation involves a rigid body movement of helix C relative to the others in the helix bundle.

Recently, Altenbach et al. [17] reported structural changes in rhodopsin associated with photoactivation. These structural changes can be interpreted in terms of movements of helices that extend into the aqueous

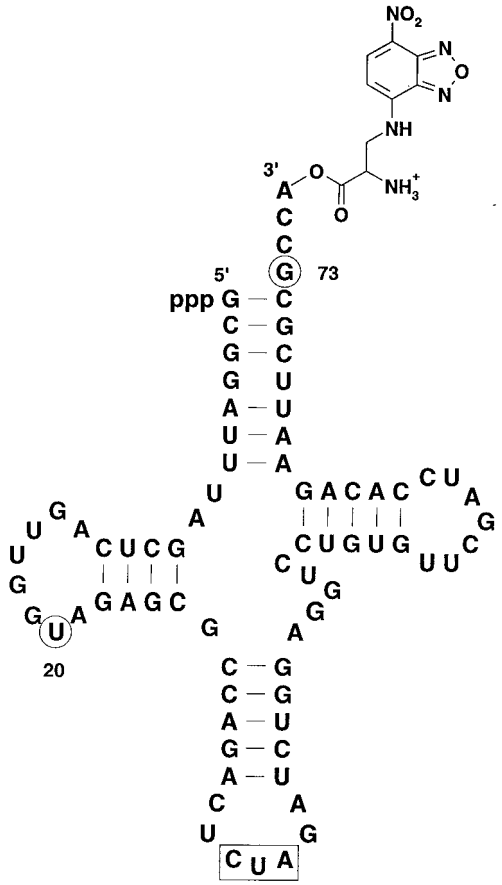


Figure 4. Cloverleaf structure of suppressor tRNA aminoacylated with NBD fluorescent unnatural amino acid. The box indicates the anticodon. Circles indicate mutations that abolish acylation by tRNA synthetases.

loop regions. Residues located along E and F helices were identified in an accompanying study as essential for transducin activation suggesting a specific structure of this interhelical loop [18]. These identified positions all lie along the inward face of the E anf F helices [17]. Large light-dependent changes were observed on the tertiary contact face of the F helix, but not on the exposed face, suggesting a rigid motion of that helix.

Helix motion was further demonstrated by an exploration of conformational changes upon activation of rhodopsin using inter-spin distance determination [19]. By construction of double cysteine mutants, each containing one cysteine at the cytoplasmic end of helix C and one cysteine at various sites in the cytoplasmic end of helix F, magnetic dipolar interactions between these spin labels were used to reveal their proximity. EPR revealed interspin distance changes upon rhodopsin photoactivation indicating a relative displacement of the helices C and F. Also, disulfide cross-linking between cysteine pairs demonstrated the proximity of these helices at the cytoplasmic end and prevented activation of transducin. The helical displacement was interpretated in terms of simple rigid body motion of helix F relative to helix C rather than secondary structural changes.

To summarize spin-labeling and time-resolved EPR investigations of helical motions in rhodopsin, the larger movements upon activation were seen in the helix F [17] and a smaller but definite motion in helix C [5, 16]. These results were interpreted in terms of a possible rigid motion of helices C and F upon activation. Interestingly, a different experimental approach, based on the construction of metal ion binding sites to block rhodopsin activation, reached the same conclusion [20]. The movement of helices C and F is involved in transducin activation. In another study, light-induced conformational changes of rhodopsin were probed by surface plasmon resonance spectroscopy [21]. Irradiation produced an increase in the thickness of the proteolipid layer consistent with elongation of the protein that could reflect protrusion of helices from the membrane.

Fluorescence studies

Investigation of ligand binding domains

Singly labeled fluorescent ligands are useful tools in defining receptor binding pockets. Because of their size and potential for modification at many positions along the sequence, peptide ligands are usually easier to label than small non-peptide organic compounds. Introduction of the fluorescent group can be achieved during peptide synthesis or post-synthesis by chemical modification of a uniquely active functional group or by selective modification. The main challenge is to modify sites that are close to or involved in the binding to the receptor while maintaining a reasonable submicromolar affinity. This can usually be achieved when starting from natural ligands with nanomolar affinities. We have prepared singly labeled fluorescent peptide analogues of substance P or neurokinin A at different positions along the sequence thus permitting to probe subdomains of binding sites [22, 23].

A panel of fluorescence spectroscopy tools is available to probe the microenvironment of the fluorophore when bound to the receptor. The intrinsic fluorescence emission properties of many dyes are sensitive

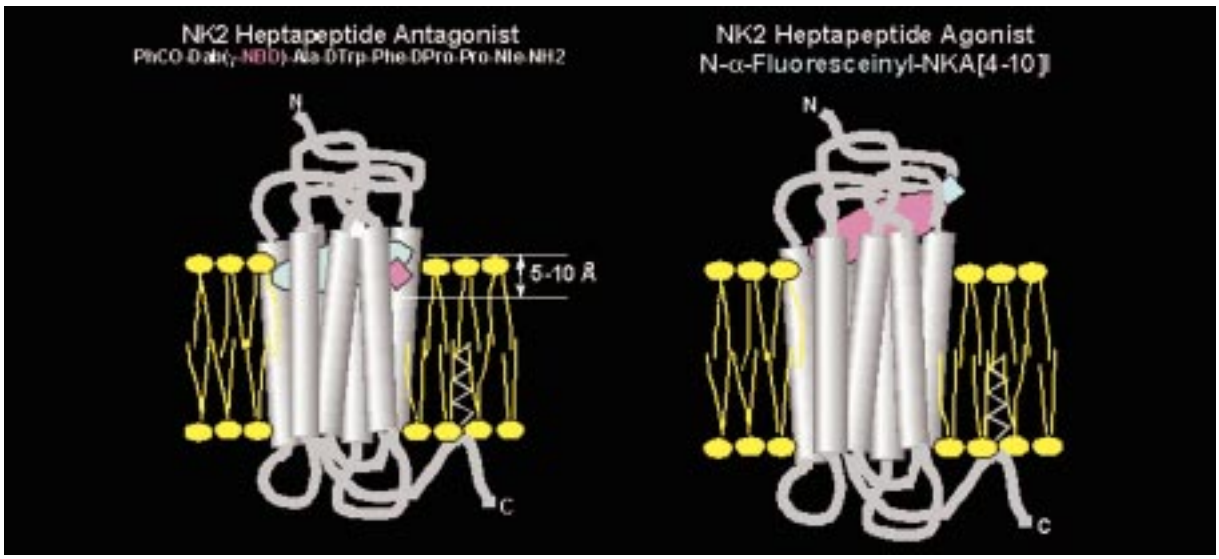


Figure 5. A schematic model comparing peptide antagonist and agonist binding to NK2.

to the hydrophobicity of the environment thus providing information on the relative polarity of binding pockets. For instance, NBD or dansyl groups have a much higher quantum yield in a low-polarity environment. This was used to unravel important differences in the relative hydrophobicity of binding sites. We have shown that the environment around the amino-terminal end of heptapeptide ligands bound to NK2 is more hydrophobic for antagonists than for agonists [22]. Likewise, using dansyl-labeled ligands we have found that the carboxy-terminal part of SP was in a more hydrophobic patch than the amino-terminal part when bound to NK1 whereas a fluorescent analogue of the non-peptide antagonist CP96,345 was in a very hydrophobic pocket [23].

The binding pocket of formyl peptide receptors was analyzed by fluorescence techniques using a panel of C-terminal fluorescein-labeled formyl peptides containing four, five or six amino acids [24]. By measuring the accessibility of fluorescein to quenching by antifluorescein antibodies for the peptides of different length, the authors suggested that the receptor binding pocket accommodates at least five but no more than six amino acids. By taking advantage of the sensitivity of fluorescein to the pH of the microenvironment, the same group investigated the nature of the specific interactions of formyl peptide ligands with the interior of the binding pocket [25]. Fluorescence quenching of formyl peptide analogs was caused by a hydrophobic interaction for a tetrapeptide, and by protonation for a pentapeptide suggesting that the formyl peptide binding pocket contains at least two microenvironments: one hydrophobic and another capable of supporting protonation, both of which can affect ligand binding. This may suggest a model in which receptor protonation may stabilize ligand binding, perhaps through a conformational change.

Collisional quenching of fluorescence is a powerful technique which can be used to probe solvent accessibility of receptor-bound ligands. Exposure to various collisional quenchers has demonstrated that the receptor-bound fluorescent β2 adrenergic antagonist carazolol is completely inaccesssible to the solvent. The failure to detect any quenching of bound carazolol fluorescence by sodium nitrite (a Förster acceptor of carazolol fluorescence with a R_0 value of 11.7 Å) indicated that the carazolol molecule is buried in the binding site of the β-AR at a depth greater than 11 Å. Moreover, analysis of the fine structure of absorption and emission spectra and fluorescence polarization indicated that carazolol is bound to the receptor in a rigid hydrophobic environment [26].

We have used the collisional quenching technique to characterize the binding pockets for NK2 and NK1 ligands [22, 23]. Several heptapeptide antagonists of structure PhCO-Xaa-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2 were labelled on position 1 (Xaa) with the environment-sensitive nitrobenzoxadiazole (NBD) probe, differing only in the length of the spacer between the NBD group and the peptide (Table 1). Collisional quenching experiments using iodide and Co^{2+} ions were performed to define the solvent acces-

sibility of the NBD group on ligands bound to NK2 receptors stably expressed in CHO cells. By comparing ligands with spacer arms of varying length, we found that the binding pocket is buried at a depth of 5–10 Å. In contrast, N-terminally NBD-labelled agonists, decapeptide NKA or heptapeptide Nle^{10}-NKA[4–10] (Table 1), bound to the NK2 receptor, were accessible to the solvent (Figure 5).

The fluorescence anisotropy of a chromophore attached to macromolecules in general, and to peptides in particular, directly reflects the molecular mobility of the fluorescent reporter group. The mobility of the peptide ligands will, upon binding to membrane receptors, be considerably restricted as compared to the free state in solution. In principle, the mobility of the ligand-attached fluorophore will to a certain extent reflect the mobility of the receptor or the ligand binding site of the receptor, the internal mobility of the ligand and finally, the motional freedom of the chromophore relative to the ligand molecule. These different kinds of motion are expected to occur in different time regimes and might be distinguished by time-resolved fluorescence anisotropy measurements to a certain extent, depending on the fluorescence lifetime of the chromophore [27]. It is the sum of all these effects which finally influences the steady-state fluorescence anisotropy. In particular, the relative mobility of the chromophore versus the peptide ligand molecule will determine the extent of the steady-state fluorescence anisotropy change upon receptor binding.

For NK2 and NK1 ligands, we have found high anisotropies for receptor-bound ligands indicating a nearly total immobilization which could be due to a densely packed environment surrounding the binding site or a conformational locking of the ligand upon binding [22, 23].

It is worth noting also that fluorescent ligands can replace radiolabeled ligands to monitor the kinetics of ligand binding and dissociation [22].

Mapping ligand-receptor interactions by FRET

We have combined fluorescent labeling of receptors by unnatural nonsense suppression mutagenesis with the technique of fluorescence resonance energy transfer (FRET) to experimentally determine distances in a model of ligand receptor interactions for the NK2 receptor [11]. FRET is a powerful technique in structural biology and dynamics for measuring distances in the 10–100 Å range [28]. In FRET, energy is transferred from a donor fluorophore to an acceptor fluorophore via a dipole interaction. The efficiency of transfer (E) depends on the inverse-sixth-power of the distance R between the donor and acceptor: $R = R_0(1/E - 1)^{1/6}$ where R_0 is the distance at which 50% of the energy is transferred. R_0 can be calculated from the properties of the fluorophores and the relative orientation of their dipole moments: $R_0 = 9.786 \times 10^3(\kappa^2 n^{-4}\Phi_D J)^{1/6}$ [Å]. κ^2 is a geometric factor that accounts for the relative orientation in space of the donor emission and acceptor absorption transition dipoles. n is the refractive index of the medium between the donor and the acceptor, Φ_D is the donor quantum yield and J is a measure of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor.

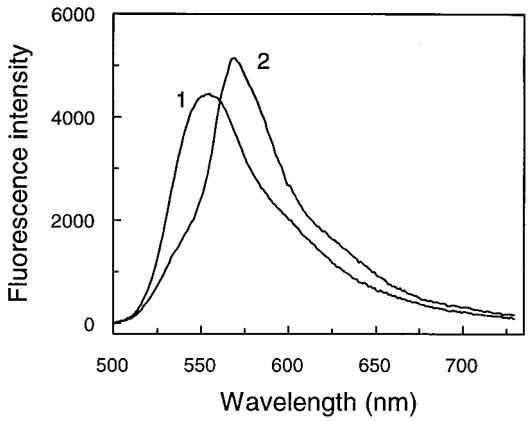
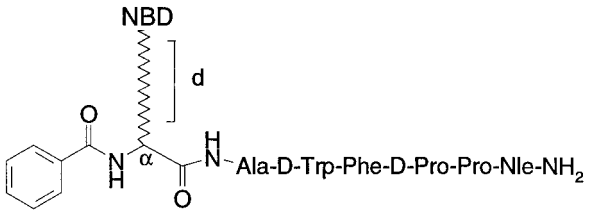


Figure 6. Representative FRET between NK2 mutant and TMR-labeled peptide antagonist. Fluorescence spectra of receptor labeled with NBD at position 233 in the absence (1) or presence (2) of TMR-labeled ligand.

Using microspectrofluorometry, we measured steady-state FRET between a tetramethylrhodamine (TMR)-labeled NK2 antagonist and different positions in NK2 mutants labeled with the fluorescence donor NBD at known sites as shown in Figure 6. Using Förster FRET theory we calculated average distances between the fluorophores. These distances were integrated into a molecular model of NK2 ligand receptor interaction (Figure 7) together with other constraints from mutational binding analysis and collisional quenching accessibility of the TMR-labeled ligand, positioning it at the water-membrane interface. FRET distances confirmed the 7 TM topology of NK2 and defined the only possible orientation of the heptapeptide ligand bound to NK2. Only one mutant at position 152 gave an inconsistent distance which could be explained by interference of the NBD group with a Trp residue at position i+4 in TM-IV or by aberrant dipole orientation. In this structural model, the

Table 1. Chemical structure of fluorescent NK2 ligands

ANTAGONISTS



Amino acid 1[a]	Distance d (Å)[b]	pK_i[c]
Ala	*n.a.*	9.81
Dab	4	8.87
Orn	5	8.84
Lys	6.5	8.83
Lys(ε-Gly)	10.5	8.85
Lys(ε-ah)	15	8.62
Lys(ε-bah)	24	8.32

AGONISTS

F*-NKA: F*-His^1-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met^{10}-NH_2
F*-Nle^{10}-NKA[4-10]: F*-Asp^4-Ser-Phe-Val-Gly-Leu-Nle^{10}-NH_2

F* = NBD or FLU

Structure[a]	pK_i[c]
NKA	8.92
NBD-Nle^{10}-NKA[4-10]	8.08
FLU-Nle^{10}-NKA[4-10]	6.51
NBD-NKA	8.23
FLU-NKA	8.80
Ac-Asp-Ser-Phe-Dap(β-NBD)-Gly-Leu-Nle-NH2	5.71

[a] Ac = acetyl; ah = 6-aminohexanoyl; bah = bis(6-aminohexanoyl); Dab = diaminobutyric acid; Dap = diaminopropionic acid; Orn = ornithine; Nle = norleucine
[b] d represents the extended distance between the NBD group and the Cα of amino acid at position 1. *n.a*: not applicable
[c] pK_i values were calculated as described [22,31].

peptide is inserted between TM-V and TM-VI, thus suggesting that antagonism may be caused by preventing correct packing of the helices required for receptor function. Interestingly, the distance differences between the TMR group and, on one hand, positions 233 and 248 in NK2, respectively, and on the other hand position 233 and the bottom of TM-V, are consistent with α-helical extensions of TM-V and TM-VI in the third intracellular loop. This supports the spin-labeling study of Altenbach et al. [17] indicating that the E-F interhelical (third intracellular) loop of rhodopsin is largely α-helical.

Ligand-induced conformational changes

Fluorescence spectrocopy has been used to monitor ligand-specific conformational changes of the β2

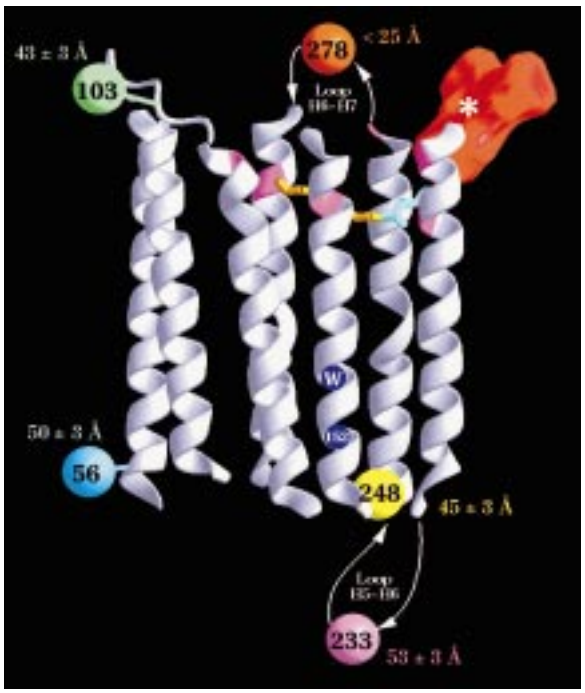


Figure 7. A molecular model of NK2 ligand-receptor interactions. The position and orientation of helices is based on the electron cryomicroscopy projection map of rhodopsin [1, 2]. FRET experimental distances between NBD-labeled sites on NK2 (colored circles with residue number) and bound heptapeptide antagonist (yellow and blue rods) labeled with TMR (red). Loops are symbolized by arrows. Positions of residues affecting binding by mutational analysis are shown in magenta.

adrenergic receptor labeled with the fluorescent probe NBD by chemical modification of native cysteines [3]. Agonist-induced conformational changes were detected from decrease in fluorescence emission from the labeled receptor. The decrease of fluorescence in response to agonists represents changes in the polarity of the environment surrounding the labeled cysteines. These changes were dose-dependent and were reversed by several adrenergic antagonists. In contrast to agonists, antagonists induced a small but reproducible increase in fluorescence intensity suggesting that antagonists may alter by themselves the receptor structure. Although the fluorescent probes were introduced at multiple sites, thereby precluding a detailed analysis of specific regions undergoing changes in conformation, this type of study demonstrates that fluorescent labels can be used as sensitive indicators of conformational changes occurring during receptor activation.

Using fluorescent NK2 mutants labeled at a single site by unnatural suppression mutagenesis, we have not detected any ligand-induced change in the fluorescence of NBD at position 248 in the NK2 receptor, a site in the C-terminal region of the third intracellular loop, in a domain putatively involved in G protein binding and activation [11]. However, quite surprisingly we detected an important decrease of the fluorescence of NK2 labeled at position 56, at the interface of TM-I and the cytoplasm, with NBD in response to antagonists (Turcatti and Chollet, unpublished results). This change in fluorescence suggests a movement of the helix I exposing the NBD side chain to a more hydrophilic environment. In the future, systematic analysis of many sites in the receptor may help map regions undergoing structural changes in GPCR in response to ligands.

Effect of palmitoylation on the GPCR structure

Fluorescent fatty acid labels have been incorporated into the C-terminal tail palmitoylation site of rhodopsin and used to probe the membrane accessibility and location of these sites in a reconstituted vesicle system [29]. Comparison of the relative effectiveness of collisional quenching of fluorescent labels by stearic acid labeled with doxyl groups at different positions allowed the determination of the membrane accessibility and disposition of the modifying fatty acids. This study indicated that the labels at the palmitoylation sites of rhodopsin are situated in the membrane. The anchoring of the palmitate in the membrane creates a fourth cytoplasmic loop that constrains the structure of rhodopsin on the intracellular face.

Conclusions and perspectives

Applications of fluorescence techniques and EPR to GPCR have recently provided insight into the receptor structure and mechanisms of ligand recognition and receptor activation at the molecular level. These techniques characterized distinct conformational and functional states of GPCR. The development and application of unnatural suppression mutagenesis to GPCR now enables us to introduce novel probes or functional groups at known sites in GPCR. In the future, application of time-resolved techniques will permit the study of rapid dynamic changes in the GPCR structure. GPCR function is modulated by the interplay of complex allosteric interactions involving at least ligand, G protein, lipids, ions and receptor-specific kinases. Biophysical techniques such as those described here or others (for instance Fourier-transformed in-

frared (FTIR) difference spectroscopy [9, 30]) will undoubtedly contribute, in combination with classical genetic and biochemical approaches, to a better understanding of these mechanisms and will help in the design of better medicines acting on GPCR.

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References

1. Schertler, G.F., Villa, C. and Henderson, R., Nature, 362 (1993) 770.
2. Schertler, G.F. and Hargrave, P.A., Proc. Natl. Acad. Sci. USA, 92 (1995) 11578.
3. Gether, U., Lin, S. and Kobilka, B.K., J. Biol. Chem., 270 (1995) 28268.
4. Resek, J.F., Farahbakhsh, Z.T., Hubbell, W.L. and Khorana, H.G., Biochemistry, 32 (1993) 12025.
5. Farahbakhsh, Z.T., Ridge, K.D., Khorana, H.G. and Hubbell, W.L., Biochemistry, 34 (1995) 8812.
6. Bain, J.D., Diala, E.S., Glabe, C.G., Wacker, D.A., Lyttle, M.H., Dix, T.A. and Chamberlin, A.R., Biochemistry, 30 (1991) 5411.
7. Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C. and Schultz, P.G., Science, 244 (1989) 182.
8. Cornish, V.W., Benson, D.R., Altenbach, C.A., Hideg, K., Hubbell, W.L. and Schultz, P.G., Proc. Natl. Acad. Sci. USA, 91 (1994) 2910.
9. Sonar, S., Lee, C.P., Coleman, M., Patel, N., Liu, X., Marti, T., Khorana, H.G., RajBhandary, U.L. and Rothschild, K.J., Nat. Struct. Biol., 1 (1994) 512.
10. Chollet, A., Turcatti, G., Nemeth, K. and Bradshaw, C., In Findlay, J.B.C. (Ed.), Membrane Protein Models, BIOS Scientific Publishers, Oxford, 1996, pp. 177–192.
11. Turcatti, G., Nemeth, K., Edgerton, M., Meseth, U., Talabot, F., Peitsch, M., Knowles, J., Vogel, H. and Chollet, A., J. Biol. Chem., 271 (1996) 19991.
12. Turcatti, G., Nemeth, K., Edgerton, M., Knowles, J., Vogel, H. and Chollet, A., Receptors Channels, 5 (1997) 201.
13. Hubbell, W.L. and Altenbach, C., Curr. Opin. Struct. Biol., 4 (1994) 566.
14. Hubbell, W.L. and Altenbach, C.S., In White, S. (Ed.), Membrane Protein Structure, Experimental Approaches, Oxford Press, Oxford, 1994, pp. 224–248.
15. Altenbach, C., Marti, T., Khorana, H.G. and Hubbell, W.L., Science, 248 (1990) 1088.
16. Farahbakhsh, Z.T., Hideg, K. and Hubbell, W.L., Science, 262 (1993) 1416.
17. Altenbach, C., Yang, K., Farrens, D.L., Farahbakhsh, Z.T., Khorana, H.G. and Hubbell, W.L., Biochemistry, 35 (1996) 12470.
18. Yang, K., Farrens, D.L., Hubbell, W.L. and Khorana, H.G., Biochemistry, 35 (1996) 12464.
19. Farahbakhsh, Z.T., Hideg, K. and Hubbell, W.L., Science, 262 (1996) 1416.
20. Sheikh, S.P., Zvyaga, T.A., Lichtarge, O., Sakmar, T.P. and Bourne, H.R., Nature, 383 (1996) 347.
21. Salamon, Z., Wang, Y., Brown, M.F., Macleod, H.A. and Tollin, G., Biochemistry, 33 (1994) 13706.
22. Turcatti, G., Vogel, H. and Chollet, A., Biochemistry, 34 (1995) 3972.
23. Turcatti, G., Zoffmann, S., Lowe III, J.A., Chassaing, G., Schwartz, T.W. and Chollet, A., J. Biol. Chem., 272 (1997) 21167.
24. Sklar, L.A., Fay, S.P., Seligmann, B.E., Freer, R.J., Muthukumaraswamy, N. and Mueller, H., Biochemistry, 29 (1990) 313.
25. Fay, S.P., Domalewski, M.D. and Sklar, L.A., Biochemistry, 32 (1993) 1627.
26. Tota, M.R. and Strader, C.D., J. Biol. Chem., 265 (1990) 16891.
27. Szabo, A.J., Chem. Phys., 81 (1984) 150.
28. Stryer, L., Annu. Rev. Biochem., 47 (1996) 819.
29. Moench, S.J., Terry, C.E. and Dewey, T.G., Biochemistry, 33 (1994) 5783.
30. Nishimura, S., Sasaki, J., Kandori, H., Matsuda, T., Fukada, Y. and Maeda, A., Biochemistry, 35 (1996) 13267.
31. Bradshaw, C.G., Ceszkowski, K., Turcatti, G., Beresford, I.J. and Chollet, A., J. Med. Chem., 37 (1994) 1991.