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Sequence analysis reveals how G protein-coupled receptors transduce the signal to the G protein

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Running Title: Entropy-variability plots for GPCRs

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Key-words: entropy-variability plots; G-protein-coupled receptors; recalcitrant residue positions; receptor evolution

ABSTRACT

Sequence entropy - variability plots based on alignments of very large numbers of sequences can indicate the location in proteins of the main active site and modulator sites. In a previous article, we applied this observation to a series of well-studied proteins and concluded that it was possible to detect most of the residues with known functional role. Here we apply the method to rhodopsin-like G protein-coupled receptors. It can be concluded that G protein binding is the main evolutionary constraint on these receptors, and that other ligands, such as agonists, act as modulators. The activation of the receptors can be described as a simple two-step process, and the residues involved in signal transduction can be identified.

Abbreviations: GPCR: G protein-coupled receptor; AGPCR: Class A GPCR; 7TM: seven transmembrane helices.

INTRODUCTION

G protein-coupled receptors (GPCRs) are involved in many biological processes and are the object of numerous research programs directed to clarify functional aspects of living organisms. GPCRs are membrane proteins consisting of a transmembrane seven-helix bundle (helices I-VII), an extracellular N-terminus, a cytosolic helix (VIII), a cytosolic C-terminus, three cytosolic loops, and three extracellular loops[1,2]. They are activated by ligands that act at the extracellular side, originating a signal that is communicated to the cytosolic side, thus activating the G protein system [3]. It has been concluded from extensive sequence analyses[4,5] that all class A GPCRs (AGPCRs) have the same gross structure, which most likely resembles that of bovine rhodopsin, the only AGPCR for which the structure is known to high-resolution[1]. This structure corresponds to the inactive form of rhodopsin and therefore the mode of activation of rhodopsin, as well as that of all other AGPCRs, remains open to speculation.

In a previous article[6], we presented new ways to analyze multiple sequence alignments where very many sequences are available using entropy-variability plots and the detection of recalcitrant residues. Since numerous sequences are indeed available for AGPCRs, we have applied these new techniques to them. These analyses shed light on the role of individual amino acids in the activation of AGPCRs.

MATERIAL AND METHODS

Sequence selection, the details of our multiple sequence alignment, entropy-variability plots, sequence weights, and recalcitrance were all explained previously[6] and will be described here only briefly. Sequences were extracted from GenBank[7], TrEMBL[8], and the GPCRDB[9] (http://www.gpcr.org/). They were aligned with a two-step, profile-based method. The Shannon entropy at position p in the multiple sequence alignment, Sp, is given by:

$$Sp = -\sum_{i=1}^{20} f_{pi} * \ln(f_{pi})$$

in which i loops over the 20 amino acid types; f_{pi} is the weighted frequency of residue type i at alignment position p; p loops over the length of the profile. The variability at position p in the multiple sequence alignment, Vp, was defined to be the number of different residue types observed at position p in at least 0.5% of the sequences. Sequence weights were used to reduce the influence of sequences that either were very similar to each other or were very different from all other sequences. The location of residues in the three dimensional structure of AGPCRs was obtained by aligning against bovine rhodopsin[1].

RESULTS AND DISCUSSION

Sequences and alignment

Much of the information about the sequences and alignments used in this study is available from the GPCRDB (http://www.gpcr.org/articles/). This website also holds results that are too voluminous to print (alignments, list of files used, profiles, residue numbering system, a list of mutation results, etc). Using 727 profiles, 1618 AGPCRs were detected in the sequence databases. The profiles were derived from the pre-aligned profiles given in the GPCRDB. These sequences were aligned as described before[6]. Figure 1 shows the sequence positions that could be aligned reliably. These are essentially only the helices. The overall average pair-wise sequence identity percentage is 23%. This is rather low, and suggests that the sequence alignment process must have been very difficult. Fortunately, the profiles for the alignment of the transmembrane helices are very selective. On top of that, each of the seven helices contains a characteristic fingerprint. Each fingerprint contains at least one residue that is very conserved throughout the entire AGPCR family, and in most cases, the few percent residues that are different are all of the same type. For example, the most common fingerprint sequence in helix VI is CWXP. In the odorant receptors the P is systematically absent, but the C is systematically present, so in this family the C provides a reliable anchor point for the alignment. The fingerprints are (subscripts indicate conservation percentage)**:** Helix I $G_{72}N_{100}$; Helix II $L_{92}XXXD_{91}$; Helix III $C_{94}(17X)L_{79}(5X)D_{80}R_{98}Y_{74}$; Helix IV W_{86}; Helix V $F_{49}(2X)P_{77}(7X)Y_{89}$; HelixVI $F_{80}(2X)C_{76}W_{58}XP_{73}$; Helix VII $N_{86}P_{97}$. Together with the rest of the transmembrane specific profiles, these fingerprints allow for a very reliable alignment. In a few difficult cases (i.e. a family in which helix IV doesn't have a W at position 420) the length and

composition of the helix aided the manual alignment.

Entropy-variability plot

Entropy, variability and recalcitrance values were calculated using the procedures described before[6]. The H-entropy used as cutoff value for recalcitrance was set at 0.015 (Fig.2). The entropy-variability plot is shown in Figure 3A. Entropy values vary widely and attain values close to the maximum, as can be expected from a series of about 1600 sequences with an average pair-wise sequence identity of 23%. As described in our study on globins, ras-like proteins, and serine proteases[6], the area of the entropy- variability plot containing the experimental points was divided in 5 boxes (11, 12, 22, 23 and 33). In the study with those three protein families, we observed the following relation between the boxes in which the residues are placed, and their function and location in the structures:

- Box 11 contains residue positions with low entropy and low variability, which are part of the main functional site (active site). Sometimes, key structural residue positions are also found in this box.
- Box 12 contains residue positions adjacent to the box 11 residue positions. In other words, they form the first shell of (mainly buried) residue positions around the active site.
- Box 22 contains core residue positions that are further away from the main functional site. They presumably have a structural role but also seem to be involved in the communication between modulators and the main site.
- Box 23 residue positions are not strictly related to any aspects of the three dimensional structure. They can be located either at the surface or in the core of the proteins. Many of them are involved in modulator interactions.
- Box 33 contains mostly surface residue positions, but also some internal positions that can be associated with modulator interactions. The problem with box 33 residue positions is that they are very variable and so some doubt remains about the correctness of the alignment that led to their identification. Most recalcitrant residues turn up in this box.

A schematic summary about functional aspects for the residue positions of these five boxes is presented in Figure 3B.

Mapping the five sectors on the structure

The high-resolution structure of bovine rhodopsin[1] reveals a 7TM bundle around an ellipsoidal central cavity formed by the helices I-III and V-VII (Fig. 4). Helix IV is not much involved in the bundle formation and makes contacts only with helix III. The broader sides of the ellipsoidal cavity are flanked by pairs of helices (II-III and VI-VII) whereas the narrower sides are flanked by helices I and V, respectively. The VI-VII pair is normal to the lipid membrane, while the II-III pair is tilted by about 30^{o}. Helix I contacts helices II and VII along its full length. On the cytosolic side, helix V contacts helices III and VI of the helix bundle, and on the extracellular side the closure is made by contacts with helix VI.

In rhodopsin, the central cavity is wide open at the cytosolic side and many of the very conserved residues that lie close to the cytosolic ends of the helices are accessible to large molecules such as G proteins. The hairpin structure connecting helices IV and V closes the cavity at the extracellular side. The central cavity seems impermeable just below the retinal binding site. At this height we find the residues that form the . This hairpin IV-V lies inside the 7TM bundle central cavity, roughly parallel to the membrane surface. It interacts with side-chains of most helices, including a disulfide bridge (Cys315-Cys480) with helix III[1]. Figure 5 shows the 3-D location of the AGPCR residue positions of the five boxes in the entropy-variability plot. Boxes 11, 12, and 22 correspond mainly to positions located in the cytosolic half of the helices that point into the central cavity. Box 23 positions are found in the central part of the helices; many of them are at the surface of the 7TM bundle; the remaining of box 23 positions are adjacent to box 22 positions forming the cytosolic side of the ligand binding pocket (retinal pocket in rhodopsin). Residues in box 33 positions are at the ends of the helices and in the cytosolic domains pointing outwards. On the extracellular side of the 7TM bundle, most of these positions are outside but some form the top (extracellular side) of the ligand binding pocket making side chain interactions with the IV-V hairpin. So far, the relation between the boxes in the entropy-variability plot and the location in the three dimensional structure agree with the conclusions drawn in a previous article[6]. One exception, however, is the presence of buried residues in the cytosolic half of helix VI, which are found in boxes 23 and 33 but located near the conserved positions of boxes 11 and 22. The functional implications of this exception will be discussed below.

The distribution of residue types on the structure

The distribution of amino acid types amongst the boxes in the entropy - variability plot of AGPCRs resembles the distribution observed for globins, ras-like proteins

and serine-proteases[6], except that residues located on the surface of the AGPCR helix bundle are all hydrophobic. Figure 6 shows the distribution of residue types in the consensus sequence over the three-dimensional structure. This distribution is asymmetric, and some of its aspects are even surprising:

- Charged residues are at the surface, pointing towards water.
- Polar residues are at the surface, pointing towards water, but a few of them are found in the helices I-III and VII pointing into the central cavity.
- Aromatic residues are mainly found in the extracellular half of the helix bundle, between the helices or pointing into the central cavity.
- Aliphatic residues are found everywhere, but there are small preferences for pointing towards the lipid or being located between the helices.
- Pro, Ala, and Gly often are found in the extracellular half of the helices I and VII, and at the extracellular ends of all helices.

The main site

Our previous study[6] showed that residue positions in box 11 of the entropy-variability plots of globins, ras-like proteins and serine-proteases form the main ligand binding or active site of these proteins whereas positions in box 12 are very near this site. We assume, as a working hypothesis, that the same will be the case for the AGPCRs. The positions in box 11 of the entropy-variability plot for AGPCRs are all known to be part of a cation-dependent allosteric site so that we have to conclude that this allosteric site is the main functional site for AGPCRs. Details about this site were recently discussed[10] using the rhodopsin structure as basis[1]. By binding to this site, sodium ions can regulate the binding of agonists[12,13].

The realization that the allosteric site is the main functional site of AGPCRs is supported by two further observations: First, the allosteric site is fully accessible to the cytosol in the inactive form of rhodopsin[1]. This allows binding of cytosolic factors such as G proteins. Second, mutagenesis studies have revealed the importance of residues in and around the allosteric site (including Tyr528, Arg340 and Tyr734) for the function of almost all AGPCR sub-families (for details see references in tinyGRAP: `http://tinyGRAP.uit.no/`[14]).

A layer of buried positions occupied by aliphatic residues is observed next to the allosteric site, all of which are found in boxes 11, 12 and 22 (most prominently, positions 133, 220, 333, 336, 344 and 732). In rhodopsin, these positions connect the allosteric site to the bottom of helix III where the extremely conserved Arg340

is located (see Fig. 5). No conclusive data have been reported regarding the functional role of these aliphatic residues[15], but our study suggests that they are involved in the transfer of signal to and from the main site.

All but one of the positions 600, 603, 604, 606, 607 and 610, near the cytosolic end of helix VI, are found in box 23 (603 sits in box 33). Most residues in this box are expected to be accessible and involved in interaction with a modulator. However, these six residues are rather buried and they are near the main site. This observation provides additional evidence for the often-suggested displacement, upon receptor activation, of the bottom of helix VI from the location observed in the rhodopsin structure, to a location where modulator interaction is possible[16-19]. Interestingly, several of these residue positions in the cytosolic half of helix VI are know to be good candidates for mutations aimed at inducing constitutive activity[14].

The agonist site

Positions in the box 23, and to a lesser extent 33, are expected to be involved in modulator interaction. Many residues of these positions are found pointing into the part of the central cavity that corresponds to the retinal pocket in rhodopsin (Fig. 5B)[1,2]. From this we conclude that the agonist must be regarded as a modulator of the main function of AGPCRs. Residue positions in box 23 line the cytosolic side of the ligand-binding pocket (retinal binding site in bovine rhodopsin). Most residue positions lining the extracellular side of the ligand-binding pocket are found in box 33. This is not too surprising, because there is a tendency for signal transduction from a modulator to the main site to go via residues in boxes 23, 22, and 12, in that order, while the residues at the extracellular side of the ligand binding pocket are not well positioned to transduce a signal in the direction of the cytosol. Together with the hairpin between the helices IV and V, and the very conserved disulfide bridge Cys315-Cys480, these residues might play a role in the structural integrity of the ligand binding pocket, and they form a barrier between the ligand and the extracellular solvent.

Box 23 and the membrane

The AGPCR 7TM bundle faces the lipid. This reduces the number of residues that are likely to be observed at these positions as Arg, Lys, Glu, Asp, Gln, Asn and His are not likely to stick into the membrane. Therefore many lipid-facing residues are observed in box 23 whereas they are functionally equivalent to box 33 residue positions in water-soluble proteins. The positions in box 23 contain more aromatic side-chains in the extracellular half of the helix bundle, but these seldom point

towards the lipid. A similar effect is seen for the hydrophobic residues in box 33 (see fig 6). No explanation has yet been found for this asymmetry.

Recalcitrant positions

Recalcitrant positions are found mainly in the cytosolic and extracellular regions and in the membrane-facing sides of helices I, IV and V, and in the extracellular halves of helices II, VI and VII (Fig. 7). In line with our previous paper[6], we can conclude that these positions are not involved in any important function. Indeed, none of the residues of these positions has been shown to be important for AGPCR function[14].

Signaling

The conventional scheme to explain the functioning of AGPCRs assumes that an agonist starts the activation by binding to a site on the extracellular side of the receptor, thereby creating a signal that crosses the membrane and reaches the cytosolic G protein [20,21]. The main site of AGPCRs is, however, formed by a cluster of conserved residues in the cytosolic half of the central cavity of the helix bundle. These residues are in direct contact with the cytosol (Fig. 5a). In our model, the main aspect of the function of AGPCRs is the interaction of this site with G proteins (or other cytosolic factors). Many AGPCRs show a basal activity, indicating that binding of an endogenous ligand is not required for activity. These ligands are merely modulators of the activity. The fact that constitutively activated mutations can easily be made corroborates this statement[22]. So, the functioning of AGPCRs can be explained by a simple two-state model[23]. In the inactive state, G proteins cannot be bound, and thus cannot be activated. In the active state, G proteins can be bound, and thus can be activated. Activation can be achieved in many ways. In principle, any shift of the equilibrium between the inactive and active states in the active direction will lead to more G protein activation. This shift may be induced by increasing the cytosolic concentrations of G proteins and salts, by decreasing the cytosolic pH, by point mutations and physiologically, by adding agonists.

AGPCR evolutionary model

All results obtained for the AGPCRs indicate that a simple two-step evolutionary model can be assumed. The first step of this model assumes that a 7TM bundle acquires the function of constitutive G-protein coupling and activation. We cannot say whether the G protein was part of the receptor or whether it already was an independent protein. In a second step, modulator sites such as the agonist-binding

site, allosteric modulator sites, phosphorylation sites, etc., evolved. These modulator sites must evolve quickly on an evolutionary time-scale, and must thus involve residues at locations that are not structurally critical, and are readily accessible to the modulator molecules. The retinal-binding pocket in rhodopsin seems ideally located to fulfill these requirements. Mutations in this site are known not to disturb the structure[24-26]. The retinal ligand can easily move between the pocket and the extracellular solvent[27]. Although we do not know how retinal gets in and out, this fact suggests that ligands of other AGPCRs can get into the pocket too, despite the presence of the IV-V hairpin. Residue positions that are spatially located between the modulator site and the main site transfer signals between these sites (see Fig. 8). These are mainly residue positions in box 22 in the entropy-variability plot. Many of the residues in box 22 have indeed been implicated in the mechanisms of receptor activation[28-32]. Studies performed after the high-resolution structure of rhodopsin became available[33-38] corroborate these ideas.

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FIGURES

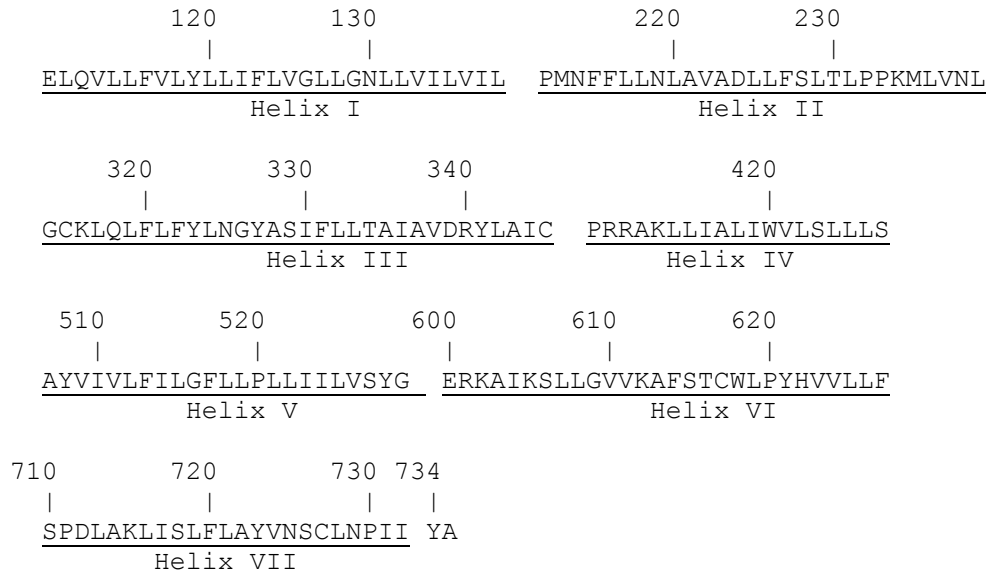

Fig. 1. Consensus sequence of AGPCRs.
The helices, as observed in the bovine rhodopsin structure[1], are shown. The numbers are global AGPCR sequence numbers used in the GPCRDB and throughout this article. Helices are underlined.

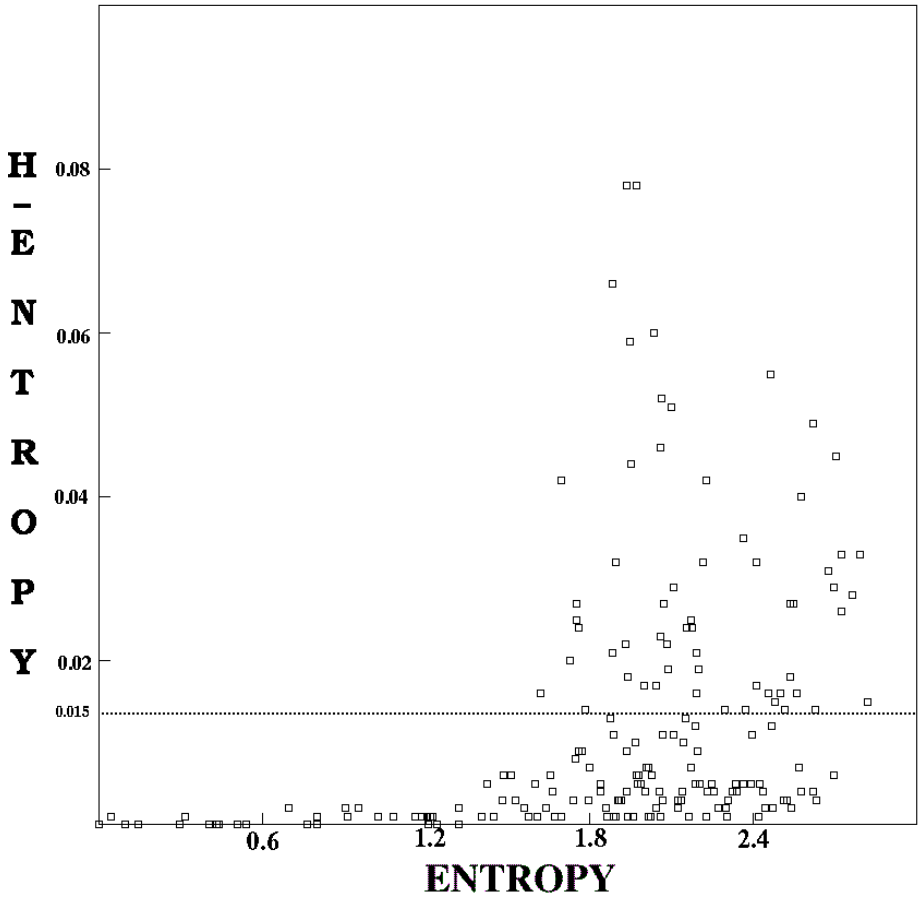

Fig. 2. H-Entropy versus Entropy plot.

The dashed line indicates the recalcitrance cut-off level (0.015).

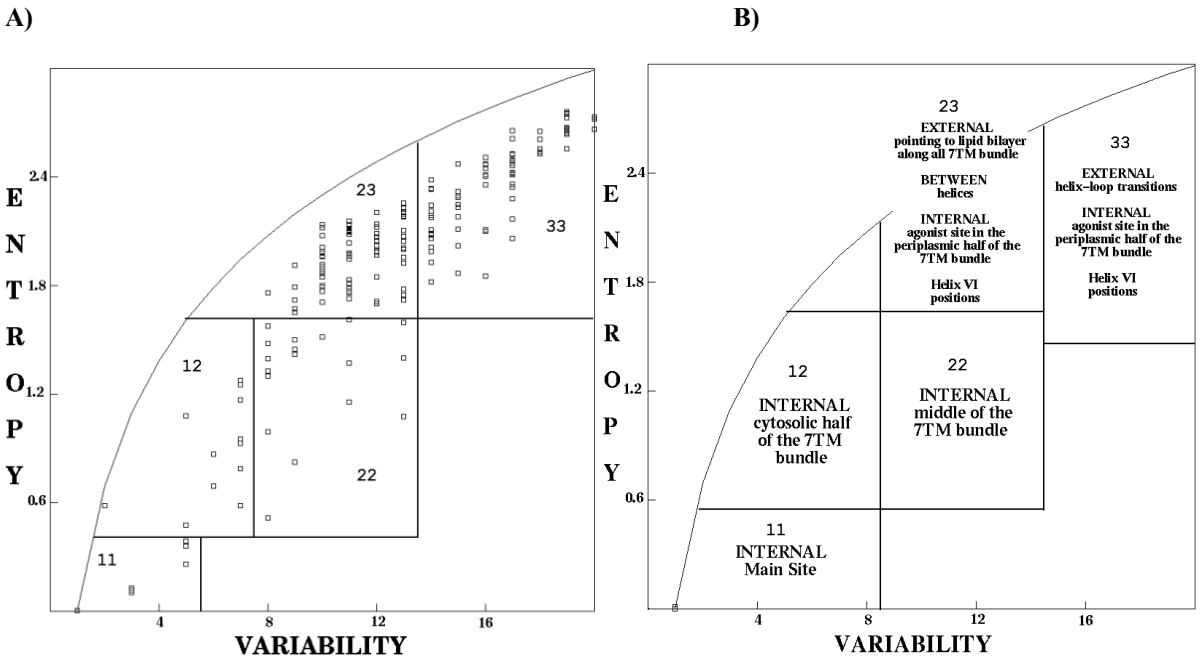

Fig. 3. Entropy versus variability plot.
A) 184 AGPCR residue positions were divided in boxes 11, 12, 22, 23, and 33.
B) Location map on the rhodopsin structure, and functional assignment of residues in the five boxes.

A) B)

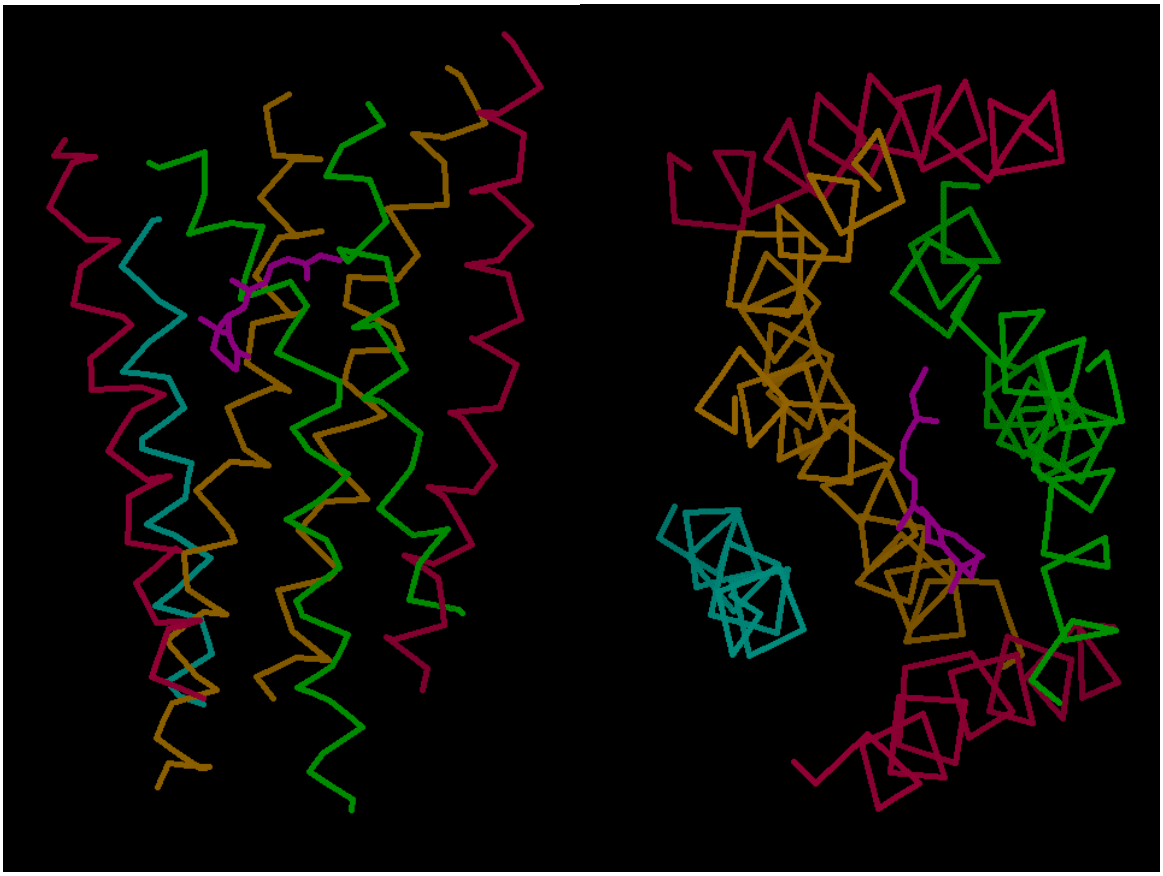

Fig. 4. The helix bundle in rhodopsin.
α-carbon trace of bovine rhodopsin. Retinal is shown in purple, helices II-III orange, VI and VII green, IV light blue, I and V red. A) side view. B) top view.

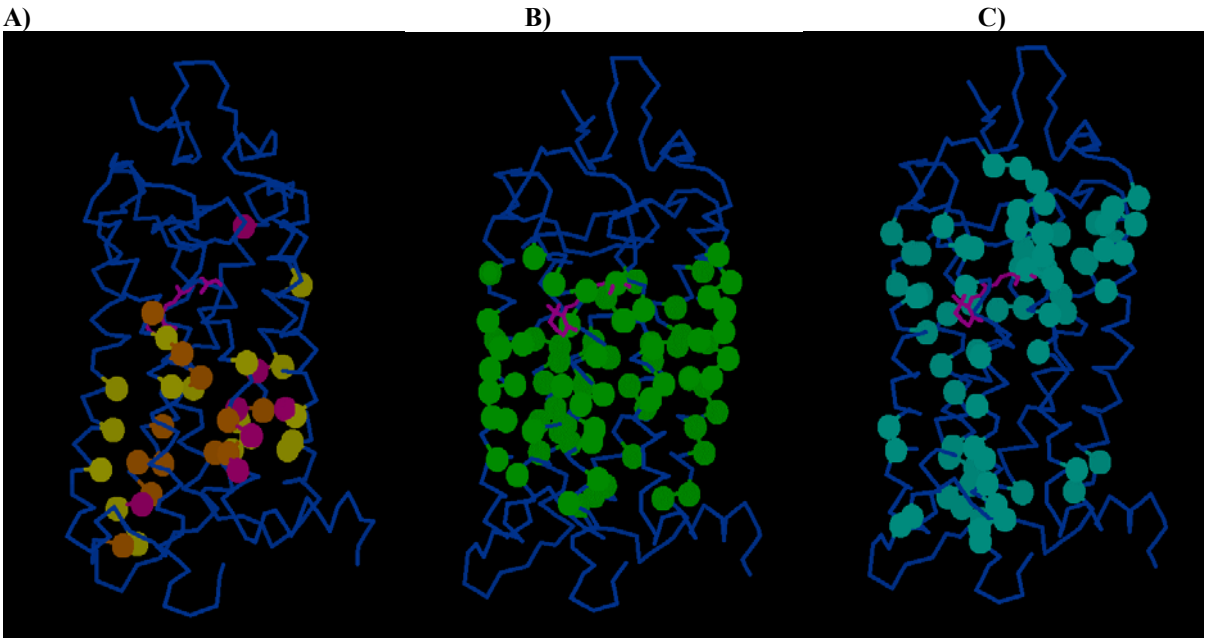

Fig. 5. α-carbon trace of bovine rhodopsin.
Retinal is shown in purple. Positions indicated in Fig. 3A are represented by coloured balls. A) red= box 11; orange=box 12; yellow=box 22. B) green=box 23. C) light blue=box 33.

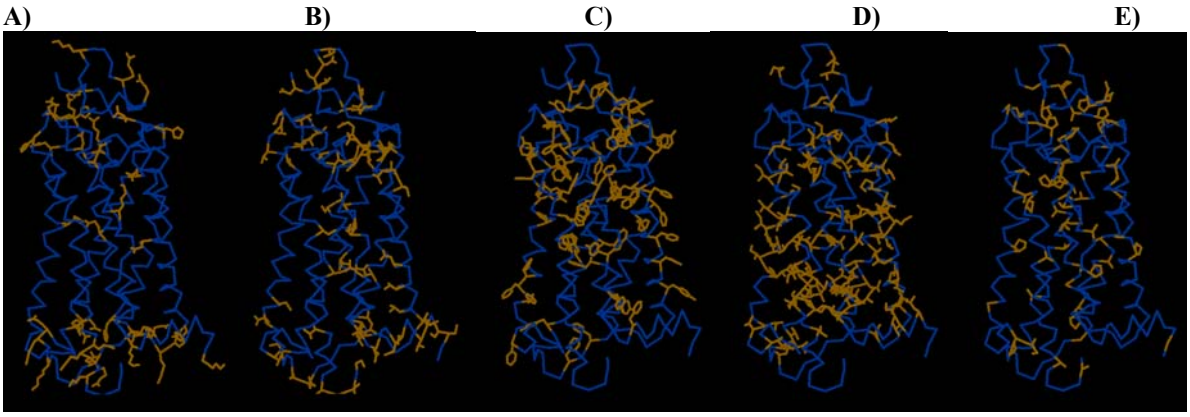

Fig. 6. Distribution of residue types in AGPCRs.
The α-carbon trace is shown in blue and residue side-chains in brown. The consensus sequence of AGPCR multiple sequence alignment is mapped on the rhodopsin structure[1]
A) charged residues. B) polar residues. C) aromatic residues. D) aliphatic residues. E) Ala, Gly and Pro.

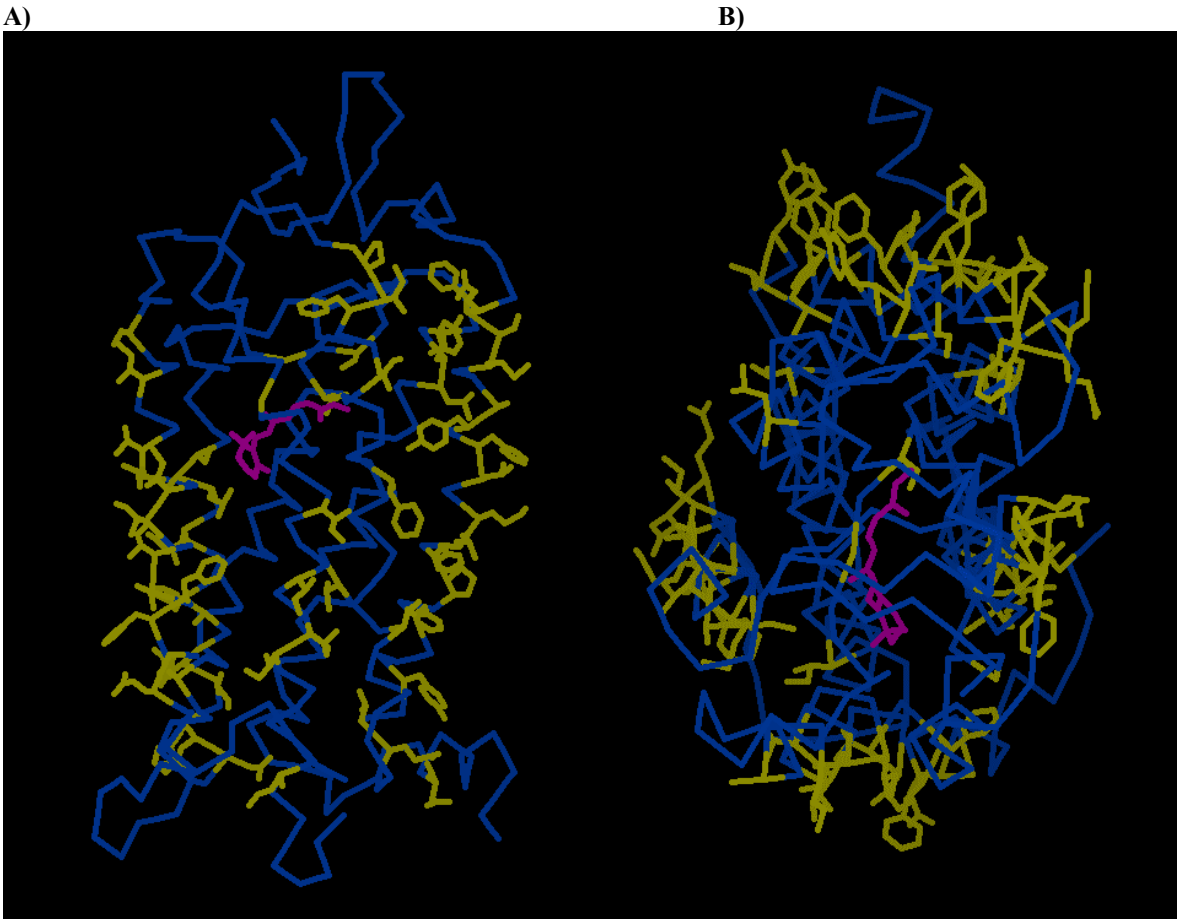

Fig 7. Distribution of recalcitrant residues.
The recalcitrant residue positions are indicated in yellow-green in the structure of bovine rhodopsin.

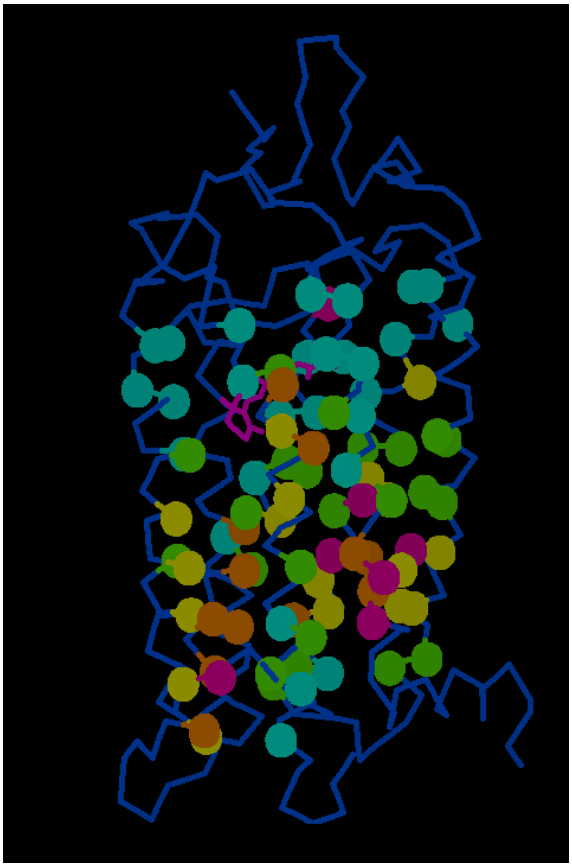

Fig. 8. Core residues positions in boxes of Fig. 3A form a signaling pathway.
α-carbon trace of rhodopsin. Retinal is in purple. Colored balls indicated classes of core residue positions. Box 11 = red. Box 12 = orange. Box 22 = yellow. Box 23 = green. Box 23 = light blue.