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Crystallizing thinking about the β2-adrenergic receptor

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Running title: Structure of β2AR

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Abstract

Two recently determined crystal structures of the human β2-adrenergic receptor (β2AR) provide a long awaited advance in the field of G protein coupled receptor (GPCR) research. The β2AR is only the second member of this, the largest family of receptors encoded in the human genome, whose structure has been solved. It follows previously determined structures of rhodopsin. Here we set these developments in historical context, discuss the daunting challenges which have been overcome, and appraise what has and has not been learned.

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For many years the study of two prototypic members of the huge family of GPCRs, the visual light "receptor" rhodopsin and the β2AR for adrenaline and noradrenaline, has guided research in this field (Lefkowitz, 2007). Now, the recent publication of two crystal structures of the human β2AR once again permits new insights to be gained from comparison of the properties of these two model seven transmembrane spanning receptors (7TMRs) (Rasmussen, et al. 2007; Cherezov, et al. 2007; Rosenbaum, et al. 2007). The remarkable and unique abundance of rhodopsin in rod outer segments (ROS) (it constitutes ~90% of the protein in ROS membranes) and its stability led to the determination of its complete amino acid sequence in 1982 by classical Edman degradation, and to the appreciation of its seven transmembrane organization (Ovchinnikov, 1982; Hargrave, et al. 1983). By comparison, the rarity of the β2AR and essentially all other GPCRs (they need to be purified several hundred thousand fold from naturally occurring sources to obtain homogeneous preparations) greatly hindered its biochemical study. Nonetheless, sufficient protein was purified so that, based on small stretches of amino acid sequence obtained from the receptor, its gene and cDNA were successfully cloned in 1986 (Dixon, et al.1986). Remarkably, in retrospect, it was only then that its close structural relationship with rhodopsin was first appreciated. This, despite the general understanding at the time that both rhodopsin and the β2AR signaled by activation of G proteins (transducin and Gs, respectively). The discovery of the homology between the β2AR and rhodopsin, followed rapidly by the cloning of additional adrenergic receptors and others, in turn triggered the rapid realization that all GPCRs share a conserved seven transmembrane organization and are members of the same gene family (Dohlman, et al. 1991).

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The first crystal structure of rhodopsin, in its inactive state was reported in 2000 (Palczewski, et al. 2000) and the new β2AR structures are the first of any other GPCR to appear. Given the close parallels and centrality of research on these two model 7TMRs it seems somehow fitting that a comparison of their molecular structures should once again be in the spotlight of molecular pharmacologists.

Why did it take so long?

Crystallization of membrane proteins, especially eukaryotic ones, remains a very difficult and time consuming process. In contrast to several thousand PDB entries for soluble proteins, only 148 unique structures of membrane proteins have been determined, of which only 40 are eukaryotic membrane proteins and only 4 of these are of human origin (www.blanco.biomol.uci.edu/Membrane_Proteins_xtal). There are multiple problems associated with crystallization of membrane proteins and GPCRs in particular. As noted, unlike rhodopsin, which is present in abundant amounts in rod outer segments and which can be purified easily, other GPCRs including the β2AR, are expressed in only tiny amounts in tissues. Therefore, isolation of sufficient amounts for crystallization purposes requires heterologous overexpression of recombinant receptors and even then substantial purification is required.

Another problem associated with crystallization of these receptors is their intrinsic conformational flexibility. In order to interact with a set of diverse ligands and transmit signals through multiple signaling pathways, 7TMRs adopt ensembles of different conformations (or different active and inactive states), which leads to conformational heterogeneity. Moreover, approximately 50% of the residues in these receptors are buried in the membrane bilayer which limits the polar surface area available for crystal contacts.

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As discussed below, the successful addressing of these problems, in addition to recent advances in crystallography, were the keys to obtaining diffraction quality crystals which finally led to the structure determination of the β2AR.

What made it possible?

In addition to high level heterologous expression of the β2AR in Sf9 cells to produce large amounts of functional receptors, two additional technical advances, one at the protein engineering level and the second at the crystallographic level, finally yielded well ordered crystals which diffracted to high resolution. Based on prior biophysical studies, the 3rd intracellular loop and the C-terminus of the β2AR appear to be the most flexible regions (Granier, et al. 2007). In addition to truncating part of the flexible C-terminus of the β2AR, two parallel approaches were taken to address this issue. First, an antibody fragment (Fab) was generated against a three-dimensional epitope corresponding to the 3rd intracellular loop (Fig. 1A) using hybridoma technology (Day, et al. 2007). This approach of co-crystallizing membrane proteins with an antibody fragment was first developed and utilized successfully in the cytochrome c oxidase structure determination and since then has been used for several other membrane proteins (Hunte and Michel, 2002). The second approach used to reduce the flexibility of the receptor and increase its polar surface area was to replace the 3rd intracellular loop with T4 lysozyme, a highly crystallizable soluble protein (Fig. 1B).

In addition to these protein engineering and antibody approaches, recent developments in membrane protein crystallography were also crucial to the success. In contrast to traditional detergent crystallization of membrane proteins, the β2AR was crystallized in either DMPC/CHAPSO bicelles or monolein lipidic cubic phase with

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cholesterol as additive. Both of these methods, which essentially rely on the use of different lipids to present a more native environment to the protein, have been reported to yield well diffracting crystals for several membrane proteins such as bacteriorhodopsin and the photosynthetic reaction center (Faham and Bowie, 2002). Moreover, as the crystals of the β2AR were small and very radiation sensitive, microfocussed beamlines were used instead of a conventional synchrotron source, both for screening and data collection.

Why the β2AR rather than others?

The large body of previously obtained biophysical and biochemical information available for the β2AR definitely helped in the design of constructs which yielded protein samples suitable for crystallization. For example, the information on the flexibility of the 3rd intracellular loop and the C-terminus of the receptor, led to strategies to stabilize and/or remove these regions. However, a feature of paramount importance and one almost unique to the β2AR was the use of a ligand affinity purification column that was developed many years ago (Caron, et al. 1979). As discussed above, one of the major obstacles in crystallizing proteins is obtaining functional and homogenous material as any heterogeneity hampers formation of well ordered crystals. Heterologously produced receptors are often purified using affinity tags genetically fused to the protein. While such purification schemes may result in reasonably pure receptors, as judged by gel analysis, there generally still exists a mixture of both functional and non-functional (i.e. denatured) receptor populations, which substantially decreases the chances of crystallization. In this regard, the alprenolol (a β-adrenergic antagonist) affinity column developed for the β2AR (Caron, et al. 1979) offers a unique advantage and yields receptor samples with

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almost 100% specific ligand binding activity and thus, a very homogenous receptor preparation. It should also be noted that carazolol, used as a ligand in both structures, has exceptionally high affinity for the receptor and very slow dissociation kinetics, thus stabilizing the receptor even under relatively harsh conditions.

How similar is the structure to that of rhodopsin?

Based on sequence conservation, GPCRs have been classified into five different classes. Rhodopsin and β2AR belong to class A, the largest and most studied of all five classes. For rhodopsin, a high-resolution structure of the full polypeptide chain is available while the β2AR structures represent truncated versions and, therefore, it is difficult to make a complete head to tail comparison. However, based on the available information, the overall topology of rhodopsin and the β2AR are quite similar. The root mean square deviation (RMSD), a measure of structural similarity between protein structures, for the alpha carbon backbone of the transmembrane region between rhodopsin and β2AR, is 1.56 Å which indicates a very similar arrangement of the transmembrane helices. This feature also supports the previous notion of a conserved activation mechanism, i.e. agonist-induced conformational rearrangement, across this class of transmembrane receptors. Nonetheless, there are structural features in the β2AR which differ significantly from rhodopsin and which thus highlight the existence of receptor-specific patterns in this class of GPCRs. For example, the 2nd extracellular loop of the β2AR contains a previously unanticipated short helix. This helix contains two disulphide bonds which appear to maintain this loop in a constrained state presumably providing space for diffusion of ligands to the binding pocket of the receptor (Fig. 1A).

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This is in contrast with rhodopsin where the 2nd extracellular loop contains a buried β-sheet which, together with the interactions of the N-terminus with other extracellular loops, forms a cap-like structure which isolates the retinal-binding site in a hydrophobic pocket.

Another interesting difference between rhodopsin and the β2AR structures is the state of the "ionic lock", defined as a network of hydrogen bonding and charge interactions between R131 in TM3 and E268 in TM6 (R135 and E247 in rhodopsin). The ionic lock is considered to maintain rhodopsin, and presumably other class A GPCRs including the β2AR, in an inactive conformation (Ballesteros, et al. 2001). Indeed the crystal structure of inactive rhodopsin displays an intact ionic lock with a distance of 2.9Å between E247 and R135, while in the light activated structure of rhodopsin the lock is broken with a distance of 4.1 Å between R135 and E247 (Palczewski, et al. 2000; Salom, et al. 2006). Both β2AR structures contain an inverse agonist carazolol, and based on classical receptor theory, one would expect such a ligand to stabilize the receptor in an inactive state, much as covalently attached retinal does for rhodopsin. However, in both β2AR structures the ionic lock is broken with a distance of 6.2 Å between R131 and E268 in the β2AR-Fab structure and 10.58 Å between R131 and E268 in the β2AR-T4 chimera structure (Fig. 1C). While one cannot exclude the possibility of alteration in some structural features of the β2AR due to binding of Fab or the presence of T4 lysozyme, the fact that similar ionic lock features were seen in both structures speaks against this being an artifact. However, the increased affinity of the β2AR-T4 chimera for agonists compared to the wild type β2AR also suggests that the receptor is in an "active

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like state" and that the broken ionic lock is a genuine feature of the receptor in the conformation which has been crystallized.

Comparison of the ligand binding pockets and the orientation of ligands between rhodopsin and the β2AR reveals another significant difference between these two receptors. Though carazolol in the β2AR and cis-retinal in rhodopsin exhibit similar binding modes, their interaction with the highly conserved "rotamer toggle" (W265 in rhodopsin and W286 in β2AR) differs. It has been suggested that a change in the rotameric state of this tryptophan in rhodopsin and other class A GPCRs serves as a second activation switch (Shi, et al. 2002). Direct interaction of cis-retinal with W265 has been proposed to maintain the inactive state of rhodospin. Although carazolol does not directly interact with W286 in the β2AR, it appears to control the rotameric state of W286 indirectly via interacting with F289 and F290. Though these interactions are proposed based on a relatively high resolution structure of the β2AR, the only direct way to validate these mechanisms would be to determine the structure of the β2AR in an active state.

Another distinctive feature is the different patterns of the two receptors with respect to their oligomeric state in the crystal structure. All the structures of rhodopsin determined so far, exhibit a receptor dimer. To the contrary, the β2AR crystals show monomers or minimal inter-receptor contacts.

What does the structure teach us?

The crystal structures of the β2AR allow a re-examination and reassessment of existing data obtained from mutagenesis and biochemical approaches. In fact much data correlating receptor mutations and ligand binding can now be explained from a structural

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perspective. There are extensive interactions observed between the receptor and the ligand carazolol (Fig. 1D & Fig. 2A). For example, D113 was previously found to be crucial for ligand binding to the β2AR. Mutation of D113 to N leads not only to complete loss of antagonist binding but also decreases the potency of agonists by several orders of magnitude (Strader, et al. 1988). Similarly, mutations of N312 result in partial or complete loss of ligand affinities (Suryanarayana and Kobilka, 1993). Indeed, both D113 and N312 are seen to interact with O17 and N19 of carazolol, thus explaining why these mutations are detrimental to ligand binding. Along the same line, V114 and F290 extensively interact with the C_8-to-C_{13} ring of the carbazole moiety of carazolol and form a hydrophobic sandwich with this aryl group. These interactions might explain the loss of affinity for aryl moiety containing antagonists (e.g. alprenolol) and agonists (e.g. epinephrine) upon mutation of V114 to Ala.

A feature of many GPCRs is that they can weakly signal even in the absence of ligand, a property referred to as constitutive activity. This has been presumed to be due to spontaneous, albeit scant isomerization of inactive receptor into the active conformation. Agonists stabilize active conformations of the receptor thus promoting cellular signaling. Some years ago it was discovered that mutation of certain residues in several adrenergic receptors greatly augmented constitutive activity of the receptors (Cotecchia, et al. 1990). Spontaneous occurrence of such mutations in a variety of receptors is now known to cause several human diseases (Spiegel, 1988). Mutations leading to enhanced constitutive activity have been thought to abrogate crucial intramolecular interactions between amino acid residues which normally constrain the receptor to its inactive state, thus somehow mimicking the effects of agonists. Mutagenesis studies had earlier

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suggested that residues at the cytoplasmic surface of TM helices 3 and 6 form the "ionic lock", discussed above, which is crucially involved in maintaining the inactive state of the receptor. As noted, the crystal structures of the β2AR accord well with these prior studies. For instance, L272 in TM6, which was the first residue identified to lead to the constitutively active phenotype of the β2AR (Samama, et al. 1993), exhibits interactions with residues towards the cytoplasmic ends of TM3 and TM5 as well as packing interactions with neighboring E268. Therefore, one can speculate that mutation of L272 may relieve the constraints at the intracellular side of the helices as well as promote disruption of the "ionic lock", thus resulting in an "active-like" state of the receptor. More importantly, several residues, which lead to CAMs (e.g. L124 & L272) and others which lead to uncoupling (UCM) (e.g. D79, N318, N322 & Y326) are linked through packing interactions i.e. certain residues which pack against residues in TM 3 and 6 responsible for constitutive activity, are also involved in interactions with residues in TM 7 which lead to uncoupling from G proteins. Thus, rearrangement of the side chains of one of these residues can affect the packing and/or orientation of others. Interestingly, these residues are also in close proximity to W286 which constitutes the rotamer "toggle switch" in the receptor. Similar to rhodopsin, a cluster of water molecules in this region is also observed which can potentially promote an extended network of hydrogen bonding interactions. It is likely that the water filled region will impose relatively low steric hindrance on the side-chains of amino acids in this region thus facilitating conformational transitions and repacking. This might be a general feature for the class A GPCRs that allows them to adopt multiple active conformations.

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What conformation is the receptor in? Does this shed light on the mechanisms of activation?

As mentioned earlier, both structures of the β2AR contain carazolol, an inverse agonist, which should stabilize the receptor in an inactive state much as covalently attached retinal does for rhodopsin. However, in contrast to the inactive structure of rhodopsin where the ionic lock is intact, both β2AR structures display a broken ionic lock (Rasmussen, et al. 2007; Cherezov, et al. 2007; Rosenbaum, et al. 2007). There are several possible explanations for this unexpected feature. As Rasmussen et al. report, carazolol reduces the basal activity of the receptor only by ~50%, which makes it a partial inverse agonist. Therefore, the first possibility is that the broken ionic lock is due to the remaining basal activity of the receptor. In fact the enhanced agonist affinity of the β2AR-T4 chimera which was observed (Rasmussen, et al. 2007) fits with this possibility. The second, more intriguing possibility is that the current structures represent an alternative "active" signaling conformation. In this context it was reported recently that carvedilol, an inverse agonist of the β2AR very similar to carazolol (Fig. 2 A & B), has no detectable G protein signaling properties but is capable of inducing β-arrestin mediated signaling (Wisler, et al. 2007). This finding suggests that the current structures may represent a β-arrestin coupled signaling state of the receptor. However, further structural and functional analysis will be required to support this interesting possibility.

What the structures do not tell us?

Many GPCRs, including the β2AR and rhodopsin, are known to exist as dimers (Milligan, 2004). While the dimeric nature of rhodopsin has been well established both by biochemical and structural data, this issue remains controversial for the β2AR. It has

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been suggested that dimers or higher order oligomers of the β2AR may regulate export of the receptor to the plasma membrane (Salahpour, et al. 2004), though receptor dimerization appears not to be essential for G protein activation (Whorton, et al. 2007). While the crystals of β2AR-Fab complex show no inter-receptor contacts, suggesting a receptor monomer, the β2AR-T4 chimera crystals do show lipid mediated contacts between helix 1 and helix 8. However, studies in native membranes have suggested helix 6 as the dimerization interface for the β2AR (Hebert, et al. 1996). Again, the possibility that the protein engineering or the Fab interferes with receptor dimerization can not be ruled out at this stage, and further structural analysis will be required to draw definite conclusions. Furthermore, as discussed above, most of the C-terminus of the receptor was truncated in order to reduce flexibility and heterogeneity. The C-terminus is known to be a site of post-translational modification as well as for interactions with proteins such as β-arrestins and, therefore, the structural organization of this region is likely to carry important information. In order to visualize this domain, a complex of the receptor with an interacting partner stabilizing this region will likely be necessary. Additionally, as the electron density of the N-terminus of the receptor is not apparent in the current structures, this region of the molecule is absent from the model and therefore features of this region remain unknown. Another important piece of information missing from these structures is the structural organization of the 3rd intracellular loop. This loop is crucial for determining the G protein coupling specificity of the receptors as well as for forming the binding interface with G proteins and other signaling partners. However in the β2AR-T4 chimera this loop is replaced by T4 lysozyme, whereas the β2AR-Fab complex contains the Fab bound to this region.

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Future directions?

The most fundamental question one can hope to answer with the receptor crystal structures is how a signal (or stimulus) recognized at the extracellular surface is transmitted to the interior of the cell leading to a specific functional outcome. However, each crystal structure represents a snapshot of only one out of many conformational ensembles or states that the receptor can adopt, thus limiting the information that can be extracted from any individual structure. Thus a major future goal will be to obtain crystal structures of the receptor in its various active states. However, considering the conformational plasticity of the activated receptor as well as the relatively low affinity and chemical instability of typical agonist ligands, this may take more than protein engineering approaches.

In this context, a paradigm shift in the field of GPCR research in the last few years has been the discovery that the receptors can signal via G protein independent mechanisms e.g. via β-arrestins (DeWire, et al. 2007). Moreover, "biased" ligands have been identified which can direct receptor signaling exclusively through either β-arrestins or G proteins. β-Arrrestin biased ligands may ultimately represent a novel class of drugs which, like conventional blockers, shut off G protein signaling, but which in addition initiate the potentially beneficial effects of β-arrestin mediated signaling. Moreover, mutant β2AR and angiotensin II receptors have been generated which are totally uncoupled from cognate G proteins but which still lead to ERK activation via β-arrestins. Determination of the structures of such mutant receptors bound to biased or unbiased ligands should help to clarify the determinants on the receptor as well as on the ligands

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that are responsible for such conformational and functional selectivity. This information will be of great value in the design of ever more selective therapeutic agents.

Another exciting and challenging goal will be the structure determination of GPCRs in complex with other signaling partners such as G proteins, β-arrestins, GRKs etc. Structural information derived from these signaling complexes will greatly aid understanding of the interaction interfaces of the receptors as well as the conformational changes in both receptor and its interacting partners that follow these interactions. Moreover, despite the fact that the signaling mechanisms of GPCRs appear to be highly conserved, receptor-specific features are also likely to exist. Thus, structural information about other class A GPCRs and those of other classes will be necessary to obtain a full understanding of these mechanisms.

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Figure legends

Figure 1: 3-Dimensional crystal structure of the human β2AR. **A.** The wild type β2AR (2-365) crystallized in complex with a Fab. **B.** An engineered β2AR-T4 lysozyme chimera. **C.** Region of the β2AR around the conserved D/ERY motif showing the ionic lock. **D.** The ligand binding pocket of β2AR with carazolol bound. Colors in the ligand (carazolol) are used to indicate oxygen (red); nitrogen (blue); carbon (yellow). The distance between R131 and E268 (ionic lock) is shown for the β2AR-T4 lysozyme chimera structure.

Figure 2: Structure and numbering scheme of two β2AR ligands, carazolol (a partial inverse agonist) and carvedilol (a β-arrestin biased ligand).

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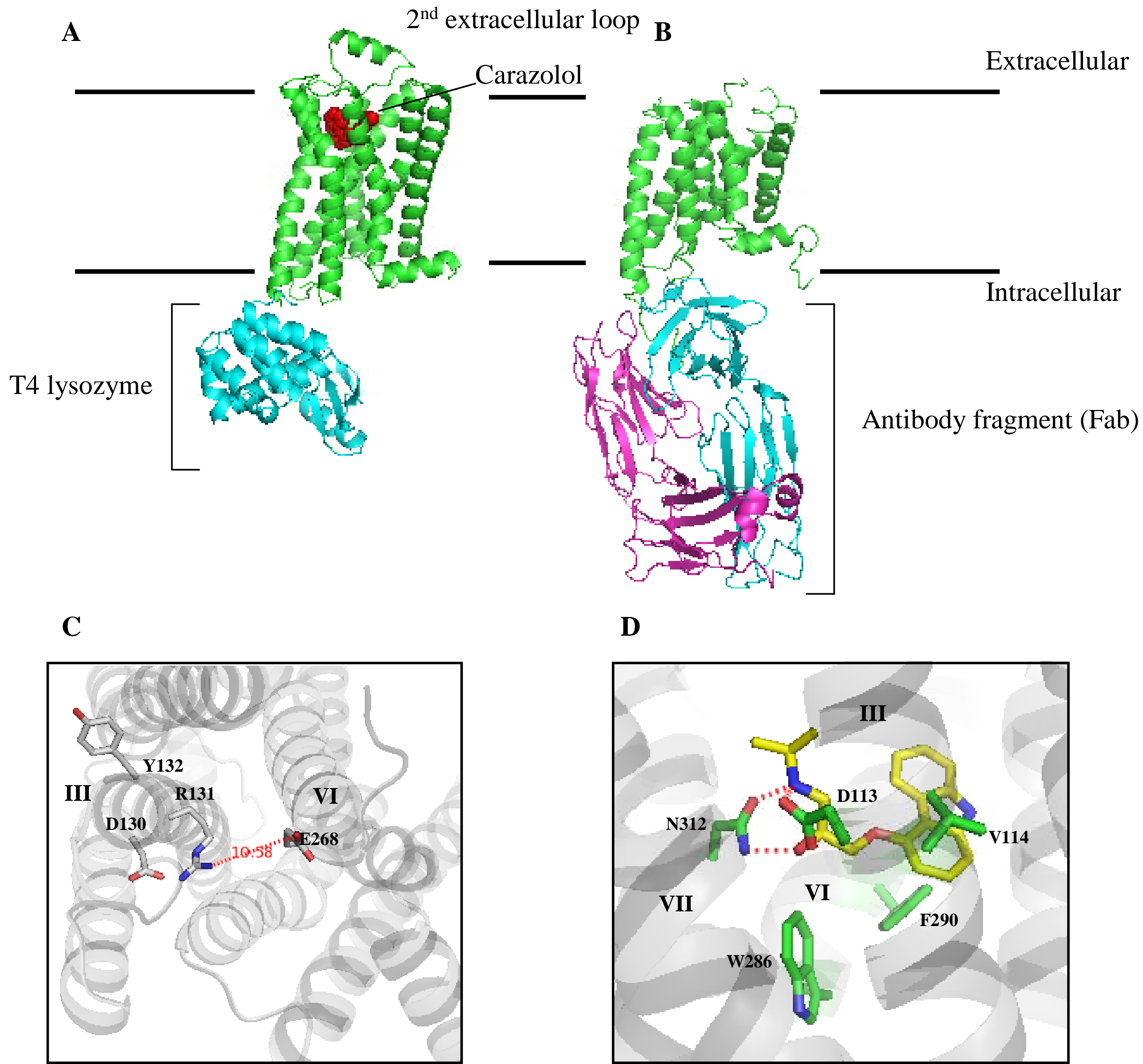

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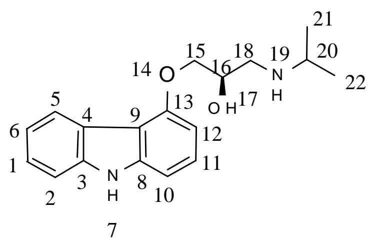
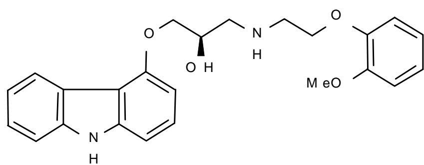
Fig. 2



Carazolol



Carvedilol

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