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ORIGINAL PAPER

Modeling and active site refinement for G protein-coupled receptors: application to the β-2 adrenergic receptor

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Abstract It is well known that G protein-coupled receptors are prime targets for drug discovery. At the present time there is only one protein from this class that has an X-ray crystal structure, bovine rhodopsin. Crystal structures of rhodopsin have become invaluable templates for the modeling of class-A G protein-coupled receptors as they likely represent the overall topology of this family of proteins. However, because of low sequence homology within the class and the inherent mobility of integral membrane proteins, it is unlikely that this single structural template reflects the ensemble of conformations accessible for any given receptor. We have devised a procedure based upon comparative modeling that uses induced fit modeling coupled with binding site expansion. The modeling protocol enables an ensemble approach to binding mode prediction. The utility of models for β-2 adrenergic receptor will be discussed.

Keywords GPCR · Protein modeling · Comparative modeling · GPCR structure

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Introduction

G protein-coupled receptors (GPCRs) are cell surface receptors that comprise one of the largest families of proteins. They are activated by a diverse set of extracellular ligands including biogenic amines, nucleosides, lipids, amino acids, peptides, and proteins [1]. Upon activation, GPCRs mediate their intracellular responses through one or more of a family of guanine nucleotide regulatory proteins (G-proteins). Several recent publications have described the role of GPCRs in drug discovery and the diversity of chemical agents that interact with this family of proteins [2]. As approximately ½ of the drugs currently marketed in the U.S. are targeted at members of this protein family with many more in development, the understanding of GPCR structure and function continues to play a critical role in the drug discovery process.

Based upon the amino acid sequence analysis of GPCRs, this family of proteins can be clustered into several distinct subfamilies (or classes) [3, 4]. Class-A GPCRs are the largest subfamily of GPCRs and are the most studied. The overall homology (compared to each other or rhodopsin) for this class of GPCR is low (on average around 20–25% sequence identity). However, there are some ''rules'' that have emerged from these analyses that suggest that there is general sequence conservation among the Class-A GPCRs [4–7]. Alignments for Class-A GPCRs provide an opportunity to infer structural and functional significance for residues based upon their levels of conservation in the sequence alignments and comparison to the available crystal structure of a GPCR, bovine rhodopsin.

There has been a myriad of computational modeling efforts to provide hypothetical models for GPCRs and

this work has recently been reviewed [8]. There are essentially two approaches to the construction of GPCR models. The first approach is based upon using comparative or homology modeling methods where the bovine rhodopsin X-ray crystal structure [9] can be used as template to construct a three-dimensional model. The second approach relies upon development of de novo methods to model GPCRs. While there are limitations to any modeling method, recent publications have shown the utility of both approaches for generating computational models for GPCRs [8, 10].

The most common approach for modeling GPCRs remains the comparative modeling approach. This method depends upon generation of meaningful sequence alignment(s) between bovine rhodopsin (the template structure) and the GPCR(s) sequence(s) of interest. Comparative modeling, sometimes called homology modeling (when the structural template and protein to be modeled share significant sequence similarity), has proven to be a successful tool for drug discovery research and most graphical software packages have implemented this technique.

Most often the success of comparative modeling is dependent upon the similarity of structural and functional relationships between the template molecule and the modeled protein. As with many other protein families, the quality of GPCR homology models has improved as better templates have become available. The early modeling used the structure of bacteriorhodopsin (BR), published in 1990 [11]. BR is a distant relative of rhodopsin and even though the sequence homology is negligible it was rationalized that BR and GPCRs would share structural features such as the seven hydrophobic membrane spanning helices connected by hydrophilic extracellular and intracellular loops. While numerous papers have been published using BR as a template for comparative modeling, ultimately the three dimensional structure of a class-A GPCR bovine rhodopsin was published by Palczewski et al. [9], PDB code 1F88 [12]. Comparison of the BR structure with the structure of rhodopsin showed significant differences. For example, the seven transmembrane helices (TM) and the interconnecting loops (intracellular and extracellular) differ in length and composition. Most importantly the highly conserved residues and motifs for Class-A GPCRs [5–7] were put in a structural context. These critical residues are not present when BR is aligned with class-A GPCRs.

Several crystallographic structures of rhodopsin are available from the Protein Data Bank ([12], http://www.rcsb.org/pdb). All structures have the same overall fold; however the higher resolution structures improve upon the positioning of water molecules, give better refinement of the retinal chromophore and ligand binding site, and include the loops and the C-terminus allowing for an essentially complete description of the protein backbone. Briefly, the sequence and corresponding structure of bovine rhodopsin has 348 amino acids, an N-terminal domain followed by an arrangement of loops connecting seven transmembrane helices (alpha and 3.10 helices), plus one additional cytoplasmic helix followed by a C-terminal intracellular domain. The structure also contains one disulfide bond between a residue in extracellular loop (EL) 2 and transmembrane helix 3. In depth comparison of rhodopsin structures and correlation of structural features with biophysical, biochemical and computational experiments have been reported [8]. Although there are low sequence similarities between bovine rhodopsin and other Class-A GPCRs, sequence analysis suggests that the Class-A GPCRs could adopt a fold similar to bovine rhodopsin [7].

The GPCR literature shows that comparative modeling of GPCRs based upon the rhodopsin structure is not routine and that each group/laboratory uses customized approaches that involve various amounts of human intervention. These include manual adjustments of sequence alignments, manual helical boundary predictions, and incorporation of iterative manual and ab initio methods for relative helix placement and loop sampling. Recent modifications to the comparative modeling approach using template molecules include incorporation of knowledge-based modeling whereby the three dimensional models for the seven individual transmembrance helices are packed using a template molecule (bacteriorhodopsin or rhodopsin) to assemble the seven helices into a bundle [13, 14]. The interhelical loops are then modeled followed by optimization of the full structure.

In recent years numerous publications describe the use of comparative based models for a large number of Class-A GPCRs including: β-adrenergic receptor, endothelin, opioid, chemokine, dopamine, vasopressin, serotonin, muscarinic [8] just to name a few. The β-2 adrenergic receptor is one of the most studied because of the plethora of experimental data available for model validation. Models of this receptor have generated using both de-novo techniques [15, 16], rhodopsin based homology modeling [17], and homology modeling combined with molecular dynamics using experimentally derived constraints [18]. Bissantz et al. [17] constructed homology models of the β2 and other Class-A GPCRs to explore the reliability of rhodopsin-based GPCR models for use in virtual screening of chemical libraries. Their models could identify known antagonists seeded in the test databases but, as

expected, the homology models were not able to identify agonists. Furse and Lybrand [15] employed de novo methods to generate models of the antagonist and agonist forms of β-2 adrenergic receptor. The Furse de novo models were somewhat similar to homology based models but they sited key structural differences that were localized to the putative ligand binding site. The Goddard group has also developed de novo methods for GPCR modeling [16]. Their work suggests that there are significant differences in agonist and antagonist binding sites. The Goddard models were validated by correlation with available mutagenesis and suggested rationale for receptor subtype specific interactions. Gouldson et al. [18] combined homology modeling with molecular dynamics to generate models of β-2 adrenergic receptor in the inactive (antagonist) and active (agonist) state. Models were tested in virtual screening and the ability of each model to select appropriate ligands. From all of these recent studies it is clear that β2 homology models derived from de novo methods or the rhodopsin template require some manipulation and optimization to be useful for drug design and molecular docking.

In this manuscript we report on a novel method for computational modeling of GPCRs that combines the best features of the comparative modeling methodology and coupled with refinement of the GPCR model so that the active site and adjacent regions adopt new conformations. We demonstrate this novel methodology by generating and refining computational models for the β-2 adrenergic receptor. The results of this methodology produce an ensemble of conformational models that were then evaluated based upon integration of mutagenesis experiments and compound structure activity relationships (SAR).

Methods

Construction of the β-2 adrenergic initial model

The comparative modeling protocol utilized in this work relies upon an accurate alignment of the β-2 adrenergic receptor with bovine rhodopsin. The amino acid sequence for the human β-2 adrenergic receptor was obtained from the Swiss-Prot database [19] (entry code P07550, ADRB2_HUMAN). In order to assess the accuracy of a pairwise sequence alignment it is possible to use highly conserved sequence motifs or fingerprints for Class-A GPCRs. Table 1 shows a list of the highly conserved residues for each of the seven transmembrane helices including the cysteines that form the conserved disulfide bond between extracellular loop 2 and TM 3 [5–7]. The sequence motifs were identified in the query sequence and the template molecule and were used as constraints in generation of the protein sequence alignment. In addition two secondary structure prediction algorithms PSIPRED [20] and SSpro [21] were used for alignment of the β-2 adrenergic receptor to bovine rhodopsin. The sequence motifs and secondary structure when combined allow for an accurate delineation of each transmembrane helix. Alignment of β-2 adrenergic receptor with bovine rhodopsin was carried out as using the automated GPCR alignment program in PRIME v1.2 (Schrodinger Inc., Portland, OR.). Following automated alignment manual inspection was made to ensure the conserved motifs, transmembrane regions, and intra- and extracellular loops were correctly aligned and insertions and deletions adjusted to maximize gap placement. Figure 1 shows that all the critical sequence motifs for Class-A GPCRs were found in the alignment of β-2 adrenergic receptor with bovine rhodopsin. The initial model of β-2 adrenergic receptor was created using PRIME version 1.5.108 (Schrodinger, LLC., Portland, OR.).

Once the initial model was created, the typical refinement includes analysis of transmembrane kinks which are often caused by the presence of proline and glycine residues. The kinks in the helices are often caused by the proline residues which do not allow formation of the "i to i + 4" hydrogen bonds that

Table 1 Sequence motifs (or fingerprints) generated from multiple alignment of Class-A GPCRs. The bovine rhodopsin transmembrane spanning helices are shown. Motifs are listed for each transmembrane helix and are shown in bold font and shaded. Key: aro = aromatic amino acid

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TM 1    WQFSMLAAYMFLLIMLGFPINFLTLYVTVQ
                              |  |
Motif                         G51 N55

TM2     PLNYILLNLAVADLFMVFGGFTTTLYTSLH
          |     |   |
Motif     N73   L79 D83

TM 3    GPTGCNLEGFFATLGGEIALWSLVVLAIERYVVVC
        |   |                   |  |  |||
Motif       C110                L128  E134/R135/Y136
        G106                        L131
TM 4    ENHAIMGVAFTWVMALACA-------
                |   |           |   |
Motif           V157            P171
                    W161            W175

TM 5    NESFVIYMFVVHFIIPLIVIFFCYGQL
           |  |          |       |
Motif      aro/aro      P215     Y223

TM 6    QKAEKEVTRMVIIMVIAFLICWLPYAGVAFYIFTH
           |                    | |
Motif      E247                 P267
                                W265
TM 7    IFMTIPAFFAKTSAVYNPVIYIMM
                              || |
Motif                         N302/P303/XX/Y306

Extracellular Loop II         C187(disulfide partner to C110)
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stabilize α-helices. For Class-A GPCRs there are three conserved motifs that can be related to helical kinks within the transmembrane helices. The three proline motifs, Pro215 of TM 5, Pro267 of TM 6 and Pro303 of TM 7 (bovine rhodopsin numbering) are conserved in the sequence alignment of rhodopsin with β-2 adrenergic receptor (Table 1 and Fig. 1). The helical bulge in TM 1 and the kink in TM 2 were not altered.

Refinement of the putative ligand binding site

The details of the molecular dynamics based expansion procedure are described in a recent manuscript (Kimura et al. manuscript in preparation). In brief, the binding site from the initial model is filled with a set of small Van-der-Wall spheres positioned on a cubic grid. Constraints are placed on the backbone of the helical

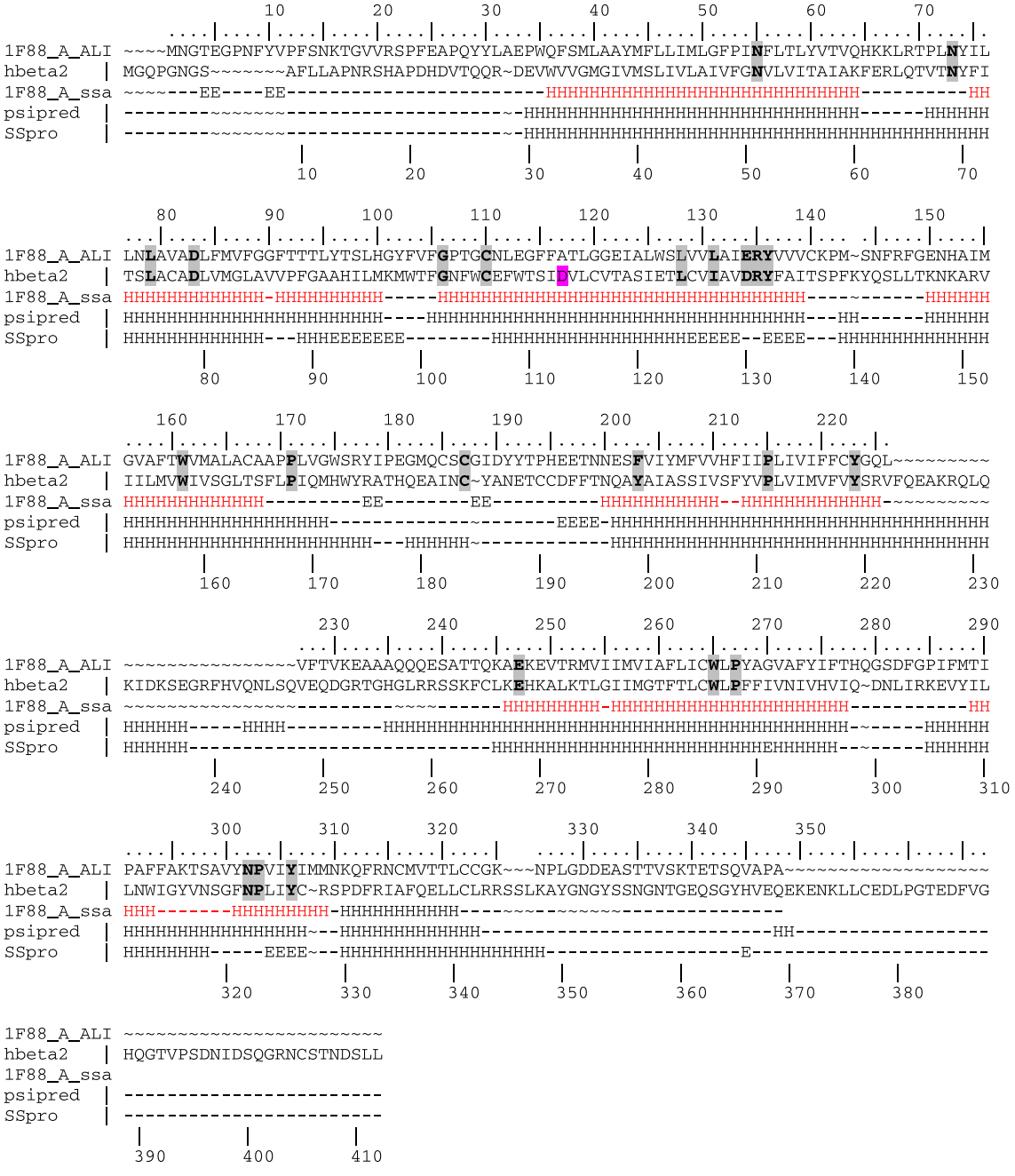

Fig. 1 Alignment of β-2 adrenergic receptor with bovine rhodopsin (PDB code 1F88). Conserved sequence motifs are shaded. Key: ssa—secondary structure derived from PDB 1F88, psipred and SSpro are secondary structure prediction algorithms. H = helix, E = strand

regions, except for the residues surrounding prolines and glycines, preventing unnatural helix deformation but allowing movement around the hinge regions. The system is placed in a lipid bilayer, solvated above and below the membrane with explicit water and equilibrated. During equilibration, the spheres efficiently pack into the shape of the ligand binding cavity. To prevent sphere evaporation, each sphere is tethered to its four nearest neighbors but to prevent shape memory, the tethers are periodically reassigned. From the equilibrated state, the van-der-Wall radii of the spheres are gradually increased in increments of 0.05 to 0.1 Å every few hundred picoseconds. Receptor states are captured from the last frame of each growth stage. A typical simulation uses starting radii of $r = 0.25$ and final radius as large as 1.0 Å.

All simulations were conducted using the NAMD package (22, NAMD was developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign), van der Waals spheres with 0.25 Å radii were originally placed on a 0.5 Å cubic grid within the cavity corresponding to the retinal binding pocket. This was done by placing spheres on a grid for the upper half of the TM helix bundle and removing all spheres overlapping with the protein. The cavity is usually well defined when the bovine rhodopsin structure is used as the template. No attempts were made to search for possible binding sites in other parts of the receptor (e.g., extracellular domains, modulator sites). In the β-2 adrenergic case, the procedure resulted in 60 van der Waals spheres. The range of sphere radii used were 0.25, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0. After staged minimization with decreasing levels of constraints, and a 100 ps heating and equilibration phase, 300 ps of simulations were conducted for each radii, each time continuing from the last set of coordinates from the previous simulation. These last coordinate frames were also captured for use in subsequent docking. Docking was conducted for 0.25, 0.3, and 0.4 stages. Beyond these stages, the pocket was too large to obtain a consensus binding mode. Because the side chain conformations from the raw receptor states are arbitrary and often high in energy, docking into a rigid protein model is not meaningful. Ligands were docked in the receptor states using the induced fit flexible receptor protocol developed by Sherman et al. [23] as implemented in Maestro (Schrodinger, Inc. Portland, Or). This allows for the simultaneous optimization of the ligand position as well as receptor side chain placement. The resulting binding modes evaluated by visual inspection.

Results and discussion

Our understanding of the structure of the binding site of GPCRs and of which residues interact with agonists and antagonists is an evolving area. While the ligand binding sites of rhodopsin have been delineated through structural and biochemical approaches, much less information is available for other GPCRs. Although no structural data for the β-2 adrenergic receptor yet exists, there is a large body of experimental data including photoaffinity labeling, fluorescence spectroscopy, site directed mutagenesis and receptor chimeras that have localized the β-2 adrenergic receptor ligand binding site in the interhelical transmembrane region at an analogous position to the retinal ligand of rhodopsin.

Site directed mutagenesis provides the strongest evidence for the location of the β-2 adrenergic receptor ligand binding site. Mutation of Asp113 in TM 3 of the β-2 adrenergic receptor was shown to be critical for agonist and antagonist binding [24]. It has been proposed in the literature that the cationic amine ligands (epinephrine, norepinephrine, dopamine, serotonin, acetylcholine) all contain a charged amine that most likely interacts with the conserved aspartate on TM 3. Mutagenesis by the Strader group has also demonstrated that two conserved serines on TM 5 can contribute to ligand specificity [25]. Ser204 and Ser207 of the β-2 adrenergic receptor have been implicated in formation of hydrogen bonds with both the meta- and para-hydroxy groups of the adrenergic ligands. Replacement of either serine with alanine reduces agonist binding by the same magnitude as removing the corresponding hydroxyl from the ligand [21]. Computer modeling of the β-2 adrenergic receptor have provided a structural rationale for these ligand/receptor interactions [26–28]. Other studies have shown that Leu64, Asp79, Asp130, Glu268, Asn312 and Ser319 also affect agonist/antagonist binding [29]. Other than Asn312, which is located in the ligand binding site, the majority of these residues do not appear to directly interact with the ligand and are not incorporated into our modeling study. Leu64, Asp130, and Glu268 are located on the intracellular side of the receptor and are likely to be involved with G-protein coupling. Asp130 and Ser319 are within hydrogen bonding distance and may stabilize the conformation of TM2 and TM7 thus impacting the overall receptor

conformation. Given the wealth of experimental data for β-2 adrenergic receptor we decided to use this GPCR as a case in point to validate our computational methodology.

The inherent flexibility of GPCRs often hinders the homology model building process as the receptor conformation from the single rhodopsin template structure may not be universally relevant. From the rhodopsin crystal structure, it has become clear that those residues conserved across the Class-A family of GPCRs are involved in interactions that stabilize the packing of the transmembrane bundle, stabilize ligand binding interactions or propagate signal transduction (Table 1). While the conservation of these residues strongly suggests that the this family shares the overall topology, in order to accommodate ligands of varying size and shape there must be subtle changes in both the helical positions and loop conformations. For example, in the rhodopsin crystal structure, extracellular loop 2 (EL2) is tightly packed against the covalently bound retinal forming the upper boundary of the ligand binding cavity. In the bovine rhodopsin structure EL2 protrudes into the transmembrane bundle significantly so that models built from this template often have severely occluded binding sites and any putative interaction between this part of the receptor and modeled ligands would require significant refinement of EL2 and the adjacent region of the ligand binding site.

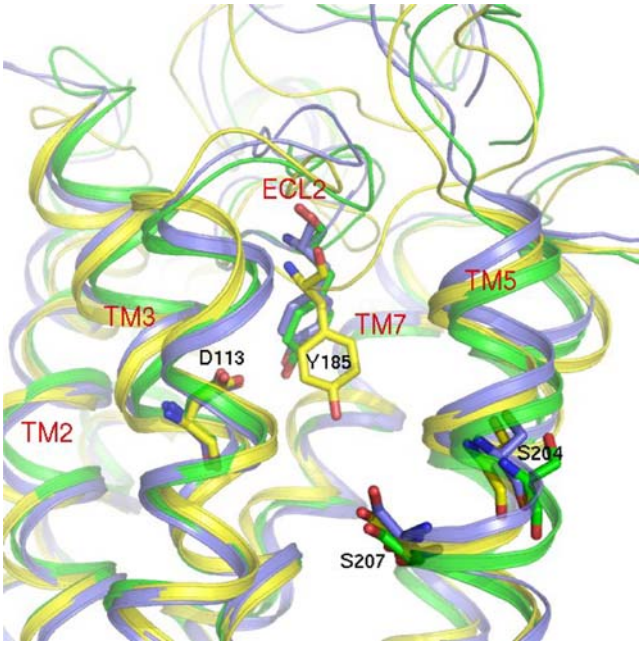

Fig. 2 Backbone trace of the initial (yellow) and two expanded states (r = 0.3, green; r = 0.5, blue) of the β-2 adrenergic receptor model. The positions of Asp113(D113), Tyr185(Y185), Ser204(S204), and Ser207(S207) are shown in stick and labeled

Our initial model of the β-2 adrenergic receptor was constructed from the rhodopsin template and included both the transmembrane segments and the loop regions. As expected, the binding site of the initial model was partially occluded by EL2, specifically by Tyr185 (Fig. 2). In this model, Tyr185 was in close proximity to Asp113 and occupied the putative ligand binding site rendering this model useless for docking studies. In the early stages of the balloon expansion procedure, Tyr185 was displaced from the ligand binding site resulting in the formation of a hydrophobic pocket that did not exist in the initial model (Fig. 2,3). It was also observed, that while EL2 had been displaced, the transmembrane bundle had contracted slightly leading to a more compact binding cavity of complementary size to the biogenic amine ligands. Further expansion of the binding site proved unproductive for agonist modeling as the distance between the key hydrogen bonding residues Asp 113, Ser 204, Ser 207, was greater than the length of the agonists. Because the size of the β2 ligands is similar to that of retinal, the β2 receptor required only minor expansion of the binding site, specifically the displacement of Tyr 185, to form a reasonable receptor conformation. However, receptors such as endothelin [30] and angiotensin II [31] bind ligands substantially larger than retinal. Examination of the size of the binding cavity for models of these receptors based on the rhodopsin crystal structure reveals that it is far too small to accommodate their ligands (data not shown). Expansion of these sites would result in a set of receptor conformations that are size compatible with the ligands and provide an unbiased set of starting points for docking studies.

Docking the agonist propanalol into the r = 0.3 state replicated the well established binding hypothesis for this ligand; the basic amine forms a salt bridge with Asp 113 and the para-catachol hydroxyl formed a hydrogen bond with Ser207 [24] (Fig. 3). In the initial model (Fig. 3a) it was apparent that the side chain rotamers of Ser204 and Asn293 oriented their hydroxyl and amide groups away from the binding cavity. The orientation of the Ser204 and Asn293 side chains were revised and propanolol redocked, resulting in a model consistent with the available mutagenesis data so that the meta-catachol hydroxyl formed a hydrogen bond with Ser204 (Fig. 3b) [24, 25]. In addition, our model predicts that the isopropyl moiety falls into the hydrophobic pocket formed by Val117, Tyr185, Tyr316, and Phe289. In our model Asn312 is involved in a hydrogen bonding network with conformations of Tyr185 and Tyr316 that are stabilized by Asn312. Asn312 stabilizes the conformation of these two residues and, as a consequence, the shape of the hydro-

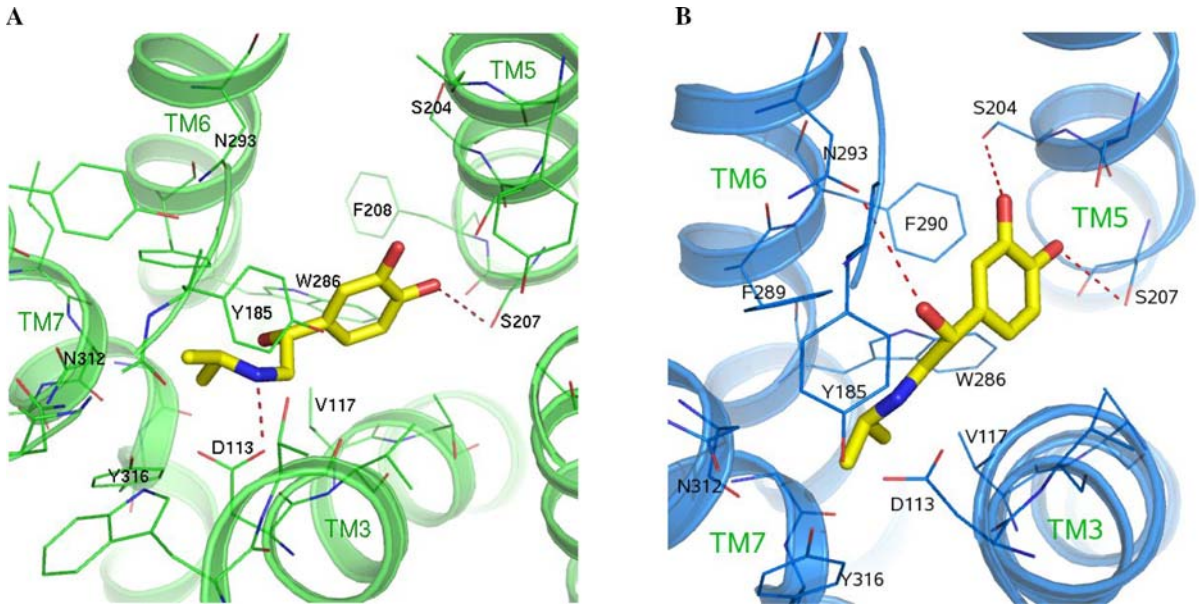

Fig. 3 Active site of β-2 adrenergic receptor with the backbone trace of the initial (A, green) and refined (B, blue) binding models of propanalol (shown in stick) in the $r = 0.3$ state. Hydrogen bonding interactions are indicated with red lines. TM helices are labeled as are receptor sidechains

phobic pocket encompassing the isopropyl moiety. The available mutational data is consistent with this model as Asn312Thr reduced agonist/antagonist binding while Asn312Phe resulted completely eliminated ligand binding [29]. In addition, the β-2 adrenergic receptor reportedly binds epinephrine with an approximately 30 to 100-fold greater affinity than it does norepinephrine. Our model would predict that the additional N-me of epinephrine inserts into the hydrophobic pocket in a manner similar to the isopropyl group of propanalol. This could provide additional interactions with the receptor or conformational constraints that may explain the increased binding affinity.

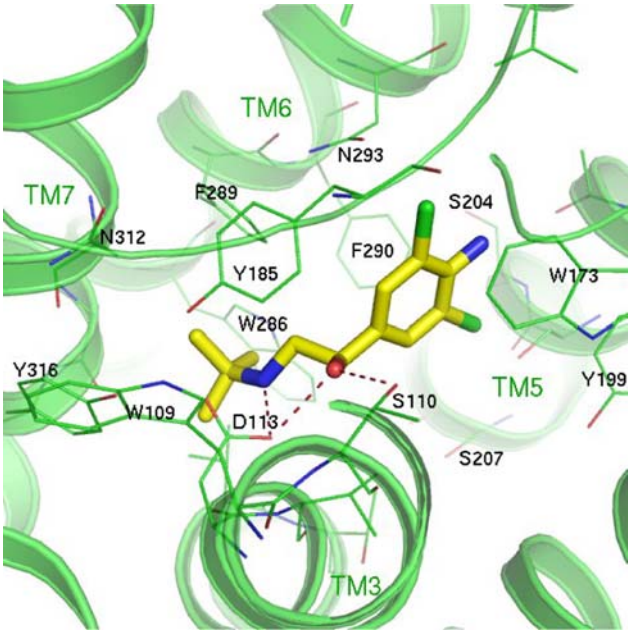

Fig. 4 Active site of β-2 adrenergic receptor with the backbone trace and the top scoring (shown in stick) and an alternate pose (green line) of clenbuterol in the r = 0.3 state

Docking of the related agoinst clenbuterol resulted in a similar orientation (Fig. 4), but one that differs in the specific interactions with aromatic moiety and the beta hydroxyl group. As with propanalol, the basic amine is predicted to form a salt bridge with Asp113, but the beta hydroxyl group forms a bidentate hydrogen bond with Asp113 and Ser110, which is located one turn above Asp113 on the β-2 adrenergic receptor model. While Ser110 mutants have not yet been reported, it has been shown that clenbuterol is not sensitive to Asn293 mutation, suggesting that its bind mode differs from propanalol. Clenbuterol does not form any hydrogen bonding interactions with TM 5. Instead the stabilizing interactions appear to be an aromatic–aromatic stack between the cholorophenyl moiety and Trp173.

Summary

GPCR receptor modeling has become a well established field which is supported by a variety of bio-

chemical and biophysical experiments that are used to confirm, refute or refine the proposed molecular models. GPCR models are often used to plan mutagenesis experiments and visualize modes of ligand binding that lead to improved ligand design. However, the quality and usefulness of the computational models depends on the assumptions made during model generation. Because GPCR model building is an inherently empirical process driven largely by the model builder, it is possible that bias can get introduced leading one to an incorrect conclusion. The key improvement of our method is that it generates an unbiased ensemble of models, that can then be evaluated in the context of the available mutatgenesis, SAR and small molecule pharmacophoric data. We have applied this technique to the β-2 adrenergic receptor beginning with initial models obtained by comparative modeling techniques using bovine rhodopsin as the template followed by model refinement using a novel protocol for binding site expansion and ligand docking to flexible binding sites using an induced fit docking protocol. While this method is able to produce models qualitatively consistent with the reported mutagenesis and ligand SAR data, they are not suitable for detailed study of ligand/receptor interactions without further refinement. Our current work is focused on refinement of these ligand/receptor complexes via molecular dynamics simulations.

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