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New vistas in GPCR 3D structure prediction

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Abstract Human G-protein coupled receptors (hGPCRs) comprise the most prominent family of validated drug targets. More than 50% of approved drugs reveal their therapeutic effects by targeting this family. Accurate models would greatly facilitate the process of drug discovery and development. However, 3-D structure prediction of GPCRs remains a challenge due to limited availability of resolved structure. The X-ray structures have been solved for only four such proteins. The identity between hGPCRs and the potential templates is mostly less than 30%, well below the level at which sequence alignment can be done regularly. In this study, we analyze a large database of human G-protein coupled receptors that are members of family A in order to optimize usage of the available crystal structures for molecular modeling of hGPCRs. On the basis of our findings in this study, we propose to regard specific parts from the *trans*-membrane domains of the reference receptor helices as appropriate template for constructing models of other GPCRs, while other residues require other techniques for their remodeling and refinement. The proposed hypothesis in the current study has been tested by modeling human β2-adrenergic receptor based on crystal structures of bovine rhodopsin (1F88) and human A2A adenosine receptor (3EML). The results have shown some improvement in the quality of the predicted models compared to Modeller software.

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Introduction

G-protein coupled receptors (GPCRs) are membrane embedded proteins that have a typical structural topology consisting of seven transmembrane helices (7TMH) connected by intracellular and extracellular loops, with an extracellular N-terminal and an intracellular C-terminal [1]. GPCRs derive their name from their ability to recruit and to regulate the activity of intracellular heterotrimeric G-proteins. Their main role is to transduce a signal across the cell membrane. Such signals emerge from interactions of GPCRs with extracellular agents, known as "ligands" or "agonists". These ligands are highly diverse entities (e.g., ions, biogenic amines, nucleosides, lipids, peptides, proteins, and even light). Ligand binding is followed by a conformational change that results in a decreased affinity of GPCR to G-proteins. Thus, the binding of such agonists and GPCRs results in signal transduction that induces a cascade of intracellular responses [2].

GPCRs are implicated in a very wide range of body functions and processes, including cardiovascular, nervous, endocrine, and immune systems. Also, their involvement in many pathological conditions, such as asthma, cardiovascular disease, central nervous system disorders, pain and others has been proven or suspected and they are considered to be the largest group of drug targets. It has been estimated that GPCRs comprise ~45% of drug targets [3] and more than 50% of current drugs are directed to GPCRs [4, 5].

The number of known GPCRs is in the thousands, and many more are being discovered as a result of recent advances in genomics and proteomics. Structures of these drug targets should be elucidated [6], in order to employ them by methods of "structure based drug design" (SBDD). The structural aspects of GPCRs are however a source of constant debate in recent years [7]. Structural information on these receptors could be attained by techniques of cryo-electron microscopy, crystallography, NMR, and computerized modeling. The predicted structures could also be validated by some other experimental techniques such as substituted cysteine accessibility method (SCAM) [8–10] and site directed mutagenesis [11, 12].

Direct experimental study of GPCR structures is currently too complicated due to their native membrane environment [13]. Only four G-protein-coupled receptors, rhodopsin, β2aR, β1aR and A2A adenosine receptor have been studied by high-resolution crystallography [14–19]. The prospects for elucidating the structures of other GPCR are not very high, and await a major breakthrough [20, 21]. With no other structures at hand, those four structures are considered to be the prototypes of the main family of GPCRs, of type A.

Due to the lack of experimental 3D-structures of other GPCRs, one could hope to gain from approximations based on molecular models. While *ab initio* modeling is not practical yet for any protein [22–24], "homology"/"comparative" modeling are quite established methods [23, 25] and are expected to be especially successful in the GPCR subfamily A that is considered to have the general features of rhodopsin [26]. Indeed, many GPCR structures have been modeled recently, based on the template of bovine rhodopsin/β2-adrenergic receptors, by using its backbone coordinates and adding the appropriate side chains of each sequence [27–31]. Such homology modeling of GPCRs has been aided mainly by experimental information from point mutations and other experimental resources [32–34]. The length of helices in the TMDs remains similar in the modeled GPCRs to those of the template receptor, and loops are not included in the template construction, except in those rare cases where loop lengths are similar to those of the template. However, other approaches for constructing models of GPCRs suggest that GPCRs could differ in their structure from rhodopsin or other known receptors even though their general features are similar [35, 36].

There are few indications to justify such deviations from the template structure, in constructing models for other GPCRs. A review by Baker and Sali [37] has shown that a homology model for a protein at medium size at least and with sequence identity of less than 30% to the template crystal structure is unreliable. The averaged sequence identity of TMDs of hGPCRs to bovine rhodopsin/β2-adrenergic receptor/β2-adrenergic receptor/A2A adenosine

receptor is lying outside the traditional homology modeling regimen. Others in the community think that this "rule" is correct in globular proteins and it is doubtful if this "rule" could be extended to membrane proteins. Also, this rule does not specify how identity should be distributed along a sequence. As much as the GPCRs superfamily is united by an overall structural topology and an ability to recruit and regulate the activity of G proteins, sequence identity between superfamily members, even in the more conserved transmembrane cores is too low. Significant sequence conservation is found, however, within several subfamilies of GPCRs. The subfamily of rhodopsin-like GPCRs is by far the largest (more than 85% of GPCRs) and is characterized by the presence of some 35 (out of ~190) highly conserved residue positions in the TMD, that may be crucial for folding and/or involved in binding and/or in activation [38].

The conserved positions along the TM sequences constitute less than 20%. In contrast, the intracellular and extracellular loops and the N- and C- terminals of GPCRs vary in their lengths and therefore they pose an alignment problem. Sequence analysis of the TMD of 302 GPCRs by Palczewski and his colleagues [39] revealed that "… the extracellular domain is the least conserved, while GPCRs display considerable conservation toward the endoplasmic side..." While this is an important observation, it lacks specific quantitative character. The conclusions of that study concentrated on individual residue conservation and on microenvironment conservation, and have thus detected the most conserved residues in the TMD. The authors concluded by suggesting that "It is reasonable to speculate that the overall fold of these receptors is highly conserved". One of the implications of that study is thus, that it is reasonable to use the overall structures of the available reference receptors to model the TMD of other GPCRs.

Therefore, the question remains open, to what extent are the structures of the four G-protein coupled receptors useful as templates for constructing models of other GPCRs? A quantitative measure of conservation in that family of GPCRs could be helpful for deciding upon the exact parts of the receptors that could be used as templates for such comparative modeling, and those that should better be excluded. Should we use the full extent of TM helices, some of the helices, or stretches of sequences along helices? It was already noticed earlier that endoplasmic parts of the TMD are more conserved than exoplasmic parts [39]. But what are the quantitative aspects of that conservation and how do they impinge on the most important decision, which is - how much of the receptors' structures may be used to model other GPCRs?

Between the two extreme approaches, to use the full crystallographic structure of the TMD of the reference receptor or to employ none of it, we propose an alternative.

From our quantitative analysis, we assign the parts of the structure of the reference receptor that may be used as a template, and suggest to construct the rest by other methods that allow deviations from the crystal structure of the template.

Methodology

In this study, we hope to examine if there is a quantitative basis for modeling the TMDs of hGPCRs based on the X-ray structures of bovine rhodopsin and/or β2-adrenergic receptor. We got a database of unique 778 rhodopsin like hGPCRs from the company of RAND Biotechnologies Ltd. They have used in-house software called GPCR-scanner to screen the protein database of human species composed of 63125 proteins (Ensembl human database). *Trans*-membrane domains allocations and multiple sequence alignments were performed by applying intelligent learning engine technology [40] from RAND Biotechnologies Ltd.

Sequence alignments

Trans-membrane domains for each of the GPCRs have been determined by TMDs-Scanner [41], and were subsequently aligned with those of rhodopsin/β2-adrenergic receptor in the crystal structure. The length of each helix was imposed by the rhodopsin template [42] and is 194 residues in total. No insertions or deletions were considered.

We use a "voting" approach, in which each amino acid contributes to the conservation at a sequence position according to its frequency in that particular position. These frequencies are measured in all sequences of the database.

$$C_{ij} = \frac{n_{ij}}{k} * 100\% \qquad (1)$$

C_{ij} is thus the conservation factor for residue type i at sequence position j.

n_{ij} is the number of sequences, which have amino acid i at position j of the multiple alignment, and k is the total number of sequences in the database.

The calculation of cumulative similarity of sequences to bovine rhodopsin/β2-adrenergic receptor or any other receptor (reference receptor) CC_l is expressed by the average of conservation scores for single sequences positions:

$$CC_l = \frac{\sum_{i=1}^{l} C_j}{l}, \qquad (2)$$

where l is the number of amino acid positions in the sequence of a helix in the TMD and Cj is the score of the reference receptor amino acid at position j and can adopt a value of 1 if the residues are identical and a value of 0 if the residues are not identical in the target-reference proteins. This score was calculated in order to evaluate the similarity for the seven TMDs separately as well as the lower endoplasmic part (G-protein binding) and the upper exoplasmic part (ligand binding) of the TMDs or over certain windows along the helices.

Optimization of windows' positions

After a window width was determined, the first residue in the helix starts the window and the identity percentage to the reference receptor was evaluated for a certain hGPCR. The window was then shifted by one amino acid all along the helix as well as the other helices. The evaluation has been performed for all the hGPCRs in our database. The analysis was done in a few windows of widths between 7–14 residues. We have concentrated on the results of windows of 11 residues, which are close to about three such turns, respectively.

Results and discussion

Conserved residues in the TMDs

Looking on the frequencies of individual residues in particular positions along the TMDs (unpublished data) reveals that large numbers of positions are enriched with a certain type of amino acid. Very low variability in specific position contents could mean importance in signal transduction pathway or in structural fold. Those residues are mostly found in the endoplasmic half of the TMDs or interacting with the membrane or phospholipids head groups in the edges of the membrane. The frequencies in some case are different from those reported by Tara Mirzadegan *et al.* [39]. For example, in helix I, Gly20, Leu23 and Val24 were found 79.2% instead of 68%; 50.2% instead of 60% and 36.6% instead of 66%, respectively. Position 9 in helix II is occupied by Leu in 92.9% while the other amino acid types are mostly very hydrophobic like Ile, Met or Phe. This position could be important to determine the height of the helix by fixing this hydrophobic moiety in interaction with the membrane. Position 16 is occupied in 43.9% by Ser or Thr which properly interact with Trp from helix IV. Basic residues are dominant in the first two positions of helix IV and helix VII. Those residues and others could play an important role in determining the orientation of the GPCR relative to the membrane.

Entire similarity in the TMDs of the hGPCRs

To check the entire similarity between all members of proteins in our database, the receptors were clustered by

requiring that clusters should be dissimilar at least by x% (with x ranging between 1–100). For example, assuming that the threshold for clustering is x%, then, if receptor A has sequence identity with receptor B less than the particular threshold, then the two receptors are considered one cluster.

The process is continued until all pairs of receptors are evaluated. Each receptor in each cluster should share sequence identity less than x% with at least one other receptor. The number of the clusters in each threshold and the shape of the obtained graph could be an index for the cumulative sequence identity within the family or subfamily. If the number of clusters converges to 1 in high threshold, then we should conclude that the cumulative sequence identity is high. Number of clusters converge to one near 25% of identity in TMDs of human GPCRs (Fig. 1) while it is in 42% and 37% of identity in amine and peptide subfamilies, respectively.

Similarities of hGPCRs with bovine rhodopsin/β2-adrenergic receptor

Similarity within the TMDs was evaluated. Higher similarity in sequences means a better chance to have close three-dimensional structures and high confidence to obtain a reliable model for the query receptor. The averaged sequence identity of TMDs of hGPCRs to bovine rhodopsin/β2-adrenergic receptor is 20.5% and 24.5%, respectively.

From pair-wise alignment of all hGPCRs with bovine rhodopsin/ β2-AR, we found 6/103 human G-protein coupled receptors respectively with sequence identity >30%. And as depicted in Fig. 2, most of the receptors
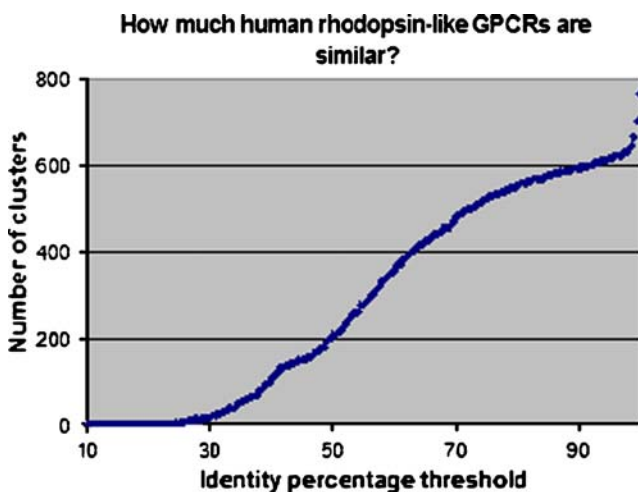
Fig. 1 Entire similarity among hGPCRs is measured by clustering. Each cluster should have at least one pair of receptors sharing percentage of identity within the TMD above a certain threshold. Number of clusters converges to one near 25% of identity. The horizontal axis shows the sequence identity threshold while the vertical one shows the number of clusters
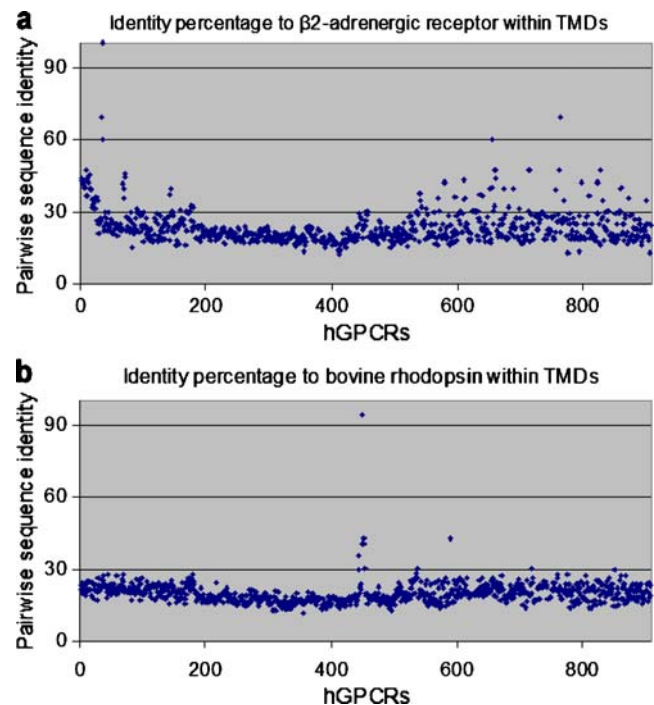

Fig. 2 Pair-wise sequence alignment of each family A receptor from human genome with rhodopsin (taking into account only TMDs). Only six receptors possess a degree of identity above 30%

have sequence identity around 20% (433 receptors have sequence identity ≥22.6% with at least one of both receptors - rhodopsin/ β2-AR). The need for a detailed analysis of the similarity to rhodopsin/ β2-AR stems from the question of usefulness of the rhodopsin/ β2-AR structures as a template for constructing other GPCRs. Any model construction must relate to sequential parts of the structure and not to individual positions in space. Therefore, it is important to record the change in the similarity along each one of the helices and to realize which parts may be considered to be "stable" enough so that a variation of sequence will not affect their structures. The conservation of sequence stretches of different length was calculated. Each stretch begins from N to C.

In this study, we employed a conservation scoring of segments in order to examine the extent of the single known GPCR structure of bovine rhodopsin which should probably not be "copied" in modeling of other GPCRs. It was shown previously that most of the conservation takes place in the endoplasmic parts of the TMD, but quantitative evaluations

Fig. 3 a–3g. Averaged identity scores (Eq. 2) over window composed ▶ of 11 residues that has been extracted from hGPCRs' *trans*-membrane domains. Horizontal axis presents initial window positions while Y-axis shows the score. The direction in each helix goes from the N-terminal side to the C-terminal side. Blue graph shows a comparison to β2-adrenergic receptor. Red squares' graph represents comparison to Bovine Rhodopsin while green triangles' graph represents comparison to consensus sequence
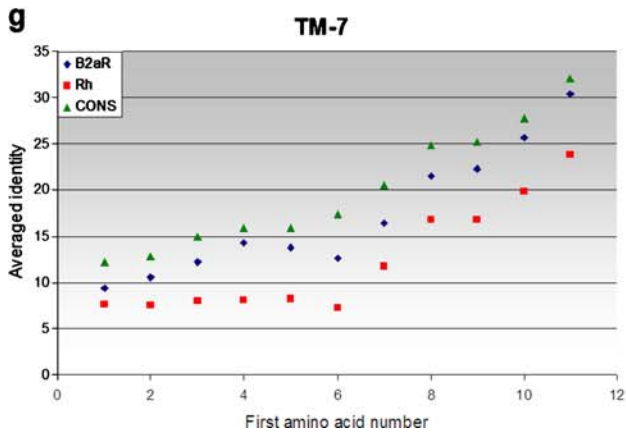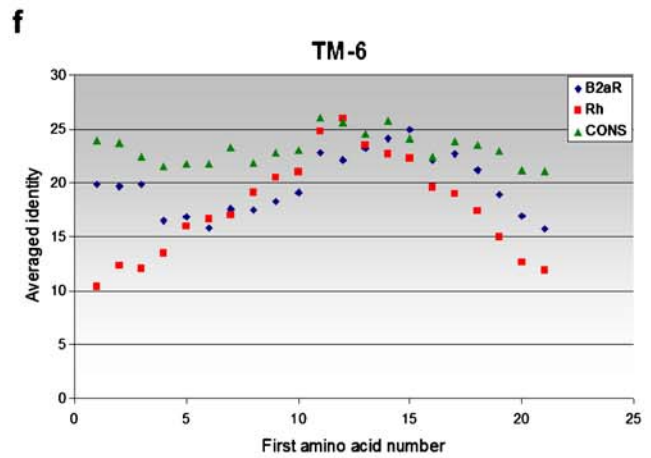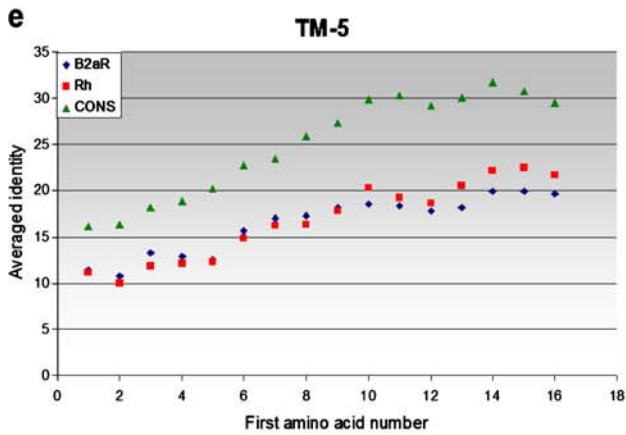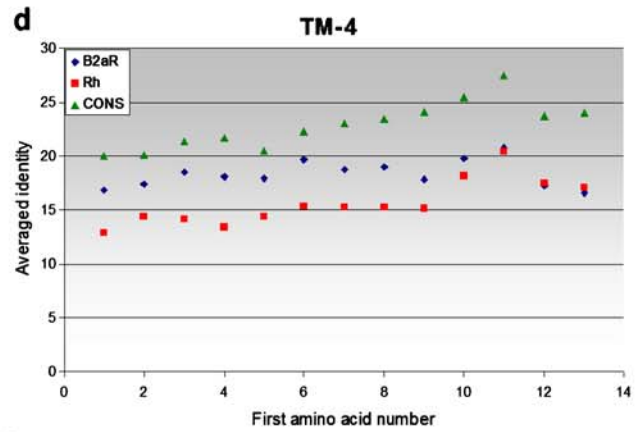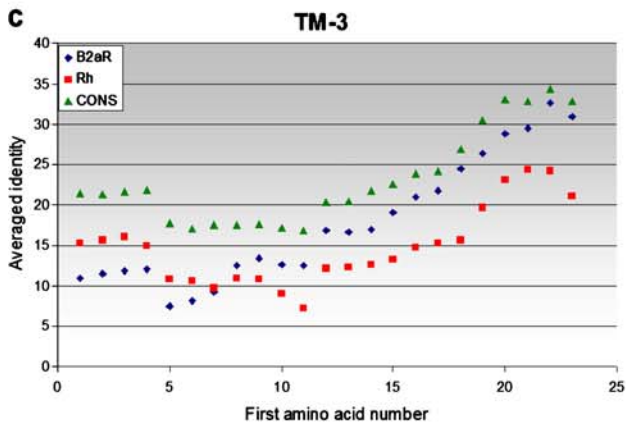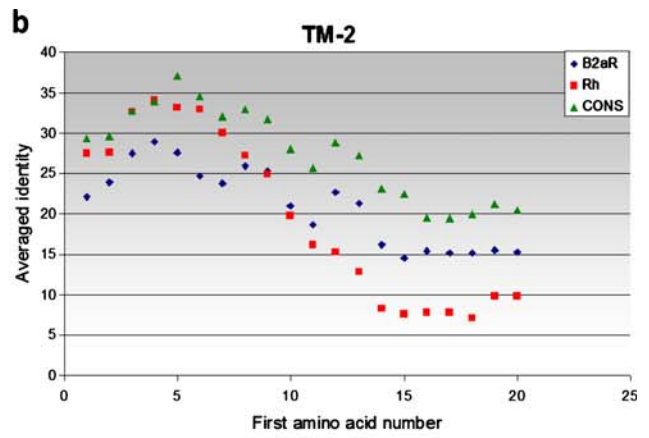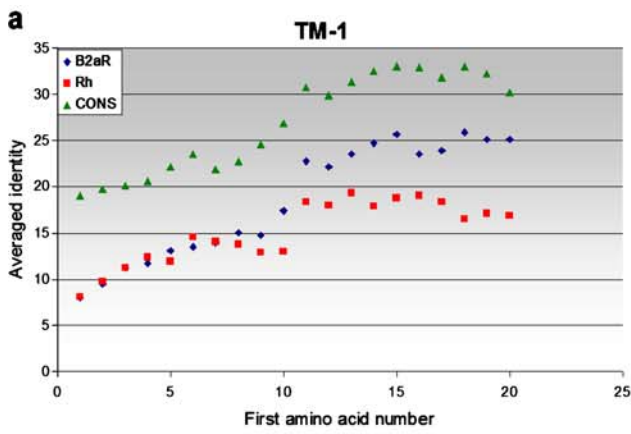
were limited to the conservations of single residues. In our study, we focused on cumulative conservation, because structural templates cannot be constructed of isolated residues that are disconnected. By computing the similarity along stretches of residues, thus constructing a "cumulative similarity", we demonstrated the quantitative aspects of the differences in conservation between the more conserved endoplasmic regions of most TM helices in rhodopsin-like hGPCRs and the exoplasmic parts. This has been attributed to the more prominent structural roles of the endoplasmic parts, or to their very similar function, to transmit a signal to intracellular G-proteins. The high variability of the exoplas-mic parts probably reflects the need to interact and to be specific to a wide range of ligands.

There are certainly other possibilities for dividing the lengths of the transmembrane helices, and these may be useful for further refinement. We have shown that it is possible to determine the exact number of residues in a "stretch" whose averaged similarity to rhodopsin/β2-AR does not exceed a certain threshold. We have also employed the "windows" method and found that then we could have better chances to model hGPCRs based on rhodopsin/β2-AR than employing the whole set of residues in the endoplasmic half (see Fig. 3a–g).

Modeling of β2-adrenergic receptor based on bovine rhodopsin and/or A2A-adenosine receptor

Since X-ray structure of β2-adrenergic receptor was released recently, we have used it to validate our findings that were obtained in this bioinformatics study. β2aR possesses a low degree of homology with Bovine Rodop-sin, the identity score in the *trans* membrane domains does not exceed 22.4%. Due to this fact many scientists claimed that rhodopsin template might not be sufficient to precisely predict the structure of β2aR as well as many other GPCRs. In this study, we decided to evaluate this opinion.

Molecular dynamics simulations: crystal structure (1F88) of bovine rhodopsin was used as a template for constructing the initial 7TM model of β2-adrenergic receptor. Dissimilar side chains of residues were added by SCWRL [43] followed by the addition of hydrogen atoms. Side chains of Aspartic acid, Glutamic acid, Argenine, Lysine and Histidine are charged. Spurious contacts were removed by 500 energy minimization steps of steep descents followed by 5000 steps of conjugate gradients. Relaxation was performed in several stages:

First stage   Hydrogen atoms were relaxed while fixing all other atoms.
Second stage   Side chains were relaxed.
Third stage   Backbone atoms of residues which are not included in the core segments were relaxed

while applying a restraining force of 10.0 kcal mol^{-1} Å^{-2} to the hydrogen bonding within the backbone.

Backbone atoms of the core segments were fixed along the whole process of 7TM model construction and optimization.

To refine our predicted model, we performed restrained MD simulations using the minimized structure as a starting point. The structure was heated to room temperature 298 K and run for 1 ns of production simulation at constant pressure (1 atm). Finally, the structure was minimized as described previously by relaxation in stages. This was repeated 50 times, generating 50 frames. The 7TM model of β2-adrenergic receptor obtained by MD simulations shows a good stereochemistry as assessed by PROCHECK [44]. The RMS deviation between the predicted model and bovine rhodopsin crystal structure was 1.95 Å. This was slightly better than the model obtained by Modeller software which was deviated by 2.1 Å.

The same approach described above was employed to predict the model of β2-adrenergic receptor based on the A2A adenosine receptor crystal structure (3EML). The obtained model has RMSD of 1.8 Å within the TMDs. The sequence identity score within the TMDs reaches 35.1% while structural alignments of the TMDs extracted from crystal structures gave RMSD of 2.1 Å. Similar value of RMSD was seen when performing structural alignment between the Modeller predicted model of β2-adrenergic receptor that was based on the A2A adenosine receptor crystal structure and crystal structure of β2AR (2RH1). It is worth noting that the core segments that were selected according to this study give a backbone RMSD of 1.39 Å between crystal structure of β2AR (2RH1) and crystal structure of bovine rhodopsin (1F88) while it is equal to 0.9 Å between crystal structure of β2AR (2RH1) and crystal structure of A2A-adenosine receptor (3EML).

Figure 4 shows the pair-wise sequence alignment of the *trans*-membrane domains of β2-adrenergic receptor and Bovine Rodopsin, while in Fig. 5, the structural alignment of the predicted 7TM model of β2-adrenergic receptor with bovine rhodopsin crystal structure is presented. The best core segments that were selected according to the findings as depicted in Fig. 3 gives backbone RMSD equal to 1.39 Å (see Fig. 6).

The shift in the cytoplasmic end of TMD-6 that has been seen in the crystal structure of β2- adrenergic receptor [16] could be explained by graph 3f. The segment of TMD-6 to be used for modeling β2-adrenergic receptor based on bovine rhodopsin in lying on the middle of the helix.

Pair-wise alignment of the TMDs of family A hGPCRs with β2-adrenergic receptor is shown in Fig. 7; 103 receptors are above 30% of identity and many others with

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Bovine Rhodopsin, TM-1    WQFSMLAAYMFLLIMLGFPINFLTLYVTVQ
Human β2AR,       TM-1    VWVVGMGIVMSLIVLAIVFGNVLVITAIAK

Bovine Rhodopsin, TM-2    PLNYILLNLAVADLFMVFGGFTTTLYTSLH
Human β2AR,       TM-2    VTNYFITSLACADLVMGLAVVPFGAAHILM

Bovine Rhodopsin, TM-3    PTGCNLEGFFATLGGEIALWSLVVLAIERYVVV
Human β2AR,       TM-3    NFWCEFWTSIDVLCVTASIETLCVIAVDRYFAI

Bovine Rhodopsin, TM-4    NHAIMGVAFTWVMALACAAPPLV
Human β2AR,       TM-4    NKARVIILMVWIVSGLTSFLPIQ

Bovine Rhodopsin, TM-5    NESFVIYMFVVHFIIPLIVIFFCYGQ
Human β2AR,       TM-5    NQAYAIASSIVSFYVPLVIMVFVYSR

Bovine Rhodopsin, TM-6    EKEVTRMVIIMVIAFLICWLPYAGVAFYIFT
Human β2AR,       TM-6    EHKALKTLGIIMGTFTLCWLPFFIVNIVHVI

Bovine Rhodopsin, TM-7    IFMTIPAFFAKTSAVYNPVIY
Human β2AR,       TM-7    EVYILLNWIGYVNSGFNPLIY
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Fig. 4 Pair-wise alignment of TMD of bovine rhodopsin with β2-adrenergic receptor

identity less than 20%. We will further test if we could obtain better models while combining segments from the two crystal structures (bovine rhodopsin and β2-adrenergic receptor).
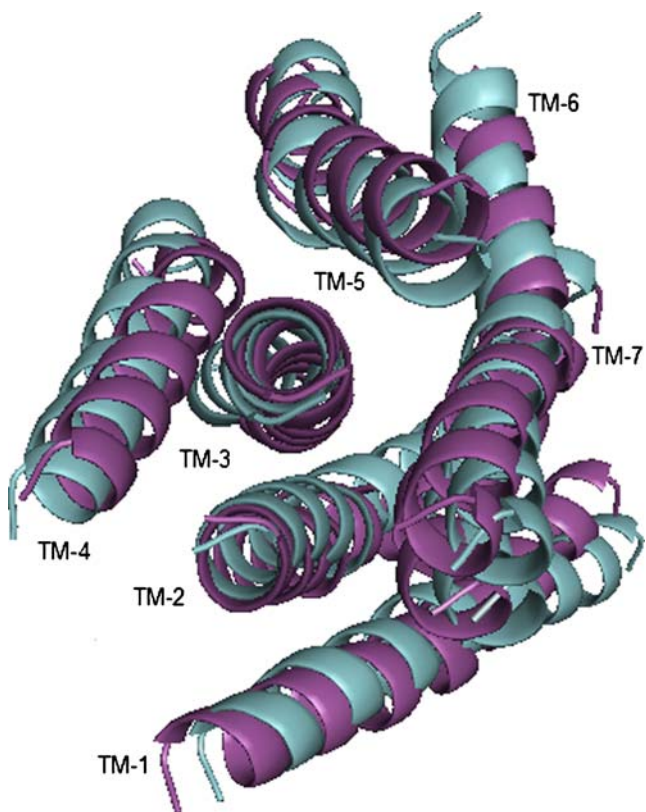

Fig. 6 Superposition of the more structurally conserved segments of the *trans*-membrane domains. Structural alignment of β2-adrenergic receptor (2RH1) colored in cyan with Bovine Rodopsin (1F88) colored in magenta. Each one of the transmembrane helices is composed of 11 residues. The selection was based on our findings from this study. The backbone RMSD is equal to 1.39 Å. The view into the membrane plane is seen from the cytoplasmic side

Conclusions

The GPCRs' solved structures might provide a unique opportunity to construct realistic models of GPCRs for drug discovery as well as for other biological purposes. However, those receptors possess a low degree of homology with many

Fig. 5 Superposition of the transmembrane helices (I–VII) of β2-adrenergic receptor (crystal structure, 2RH1) with β2AR model constructed based on Bovine Rodopsin (1F88) as template. The backbone root mean square deviation (RMSD) is equal to 1.95 Å. Crystal structure of β2AR is shown in cyan color while predicted model is shown in magenta color. In general, the upper half is more deviated than the lower half. The view into the membrane plane is seen from the cytoplasmic side
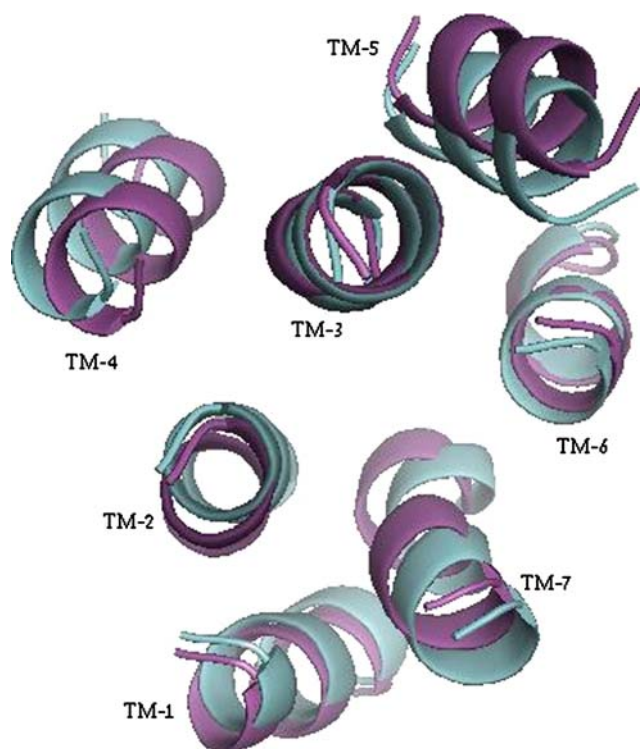

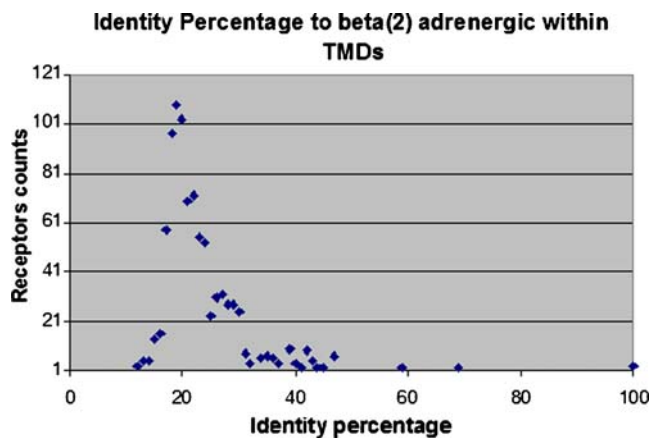**Fig. 7** Pair-wise alignment of each family A receptor in the human genome with β2-adrenergic receptor separately (only TMDs). 103 receptors possess a degree of identity above 30% threshold

GPCRs, and many experts in the field believe that such templates might not be sufficient to accurately predict the structure of those remote homologues GPCRs. In this study, we decided to evaluate this opinion and performed a qualitative and a quantitative analysis of family A hGPCRs database. We verified the usefulness of employing crystal structure of solved GPCRs as a template for modeling the TMDs of other receptors from the same family. In most cases, as shown in Fig. 3a–g, helix terminals display a smaller conservation than other parts of the helices. These variations could be connected to the structural changes from helix to loop at both the endoplasmic and exoplasmic terminals. Conservation is mostly more obvious in the endoplasmic region (except for helix VI that has a larger conservation value at the middle). Structural analysis of β2-adrenergic receptor compared to Bovine Rhodopsin has shown a shift in the endoplasmic end of TMD-6. This experimental finding is well explained based on results of this study.

The construction of more accurate models of hGPCRs is possible and requires refinements of the primary models with molecular dynamics and/or simulated annealing while putting restraints on the core segments. As well, based on the information extracted by this study, we are planning to use iterative stochastic elimination [45] (ISE) technique in order to construct better models for unsolved GPCRs, starting with a partial template of solved GPCR.

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