Research Article

Residue conservation and dimer-interface analysis of olfactory receptor molecular models

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Abstract

Olfactory Receptors (ORs) are members of the Class A rhodopsin like G-protein coupled receptors (GPCRs) which are the initial players in the signal transduction cascade, leading to the generation of nerve impulses transmitted to the brain and resulting in the detection of odorant molecules. Despite the accumulation of thousands of olfactory receptor sequences, no crystal structures of ORs are known to date. However, the recent availability of crystallographic models of a few GPCRs allows us to generate homology models of ORs and analyze their amino acid patterns, as there is a huge diversity in OR sequences. In this study, we have generated three-dimensional models of 100 representative ORs from *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Sacharomyces cerevisiae* which were selected on the basis of a composite classification scheme and phylogenetic analysis. The crystal structure of bovine rhodopsin was used as a template and it was found that the full-length models have more than 90% of their residues in allowed regions of the Ramachandran plot. The structures were further used for analysis of conserved residues in the transmembrane and extracellular loop regions in order to identify functionally important residues. Several ORs are known to be functional as dimers and hence dimer interfaces were predicted for OR models to analyse their oligomeric functional state.

Introduction

The discovery of olfactory receptors (ORs) by Buck and Axel (1991), nearly two decades ago, has greatly helped us in understanding olfactory function. The knowledge of these receptors not only provided information about their function and organization, but also served as a starting point to understand other chemosensory receptors in mammals.

Mammals have to recognize and discriminate a multitude of odour molecules. This is important to find food, identify mates and offsprings and avoid danger. This is accomplished by an elaborate olfactory system composed of the main olfactory epithelium, the vermonasal organ, the septal organ and the Grueneberg ganglion (Breer *et al.* 2006). The first step in recognizing the odour is the interaction of odour molecules with ORs in the nose. Chemoreception in insects and nematodes has long been a major focus of insect ecology. With the use of bioinformatics, candidate receptor proteins mediating olfaction were identified from the genome of *Drosophila melanogaster*. The functional organization of olfactory system is remarkably similar in organisms ranging from insects to mammals; hence the principles elucidated in one experimental organism often apply to many others.

Rather than binding specific ligands like most receptors, ORs display affinity for a range of odour molecules, and conversely a single odorant molecule may bind to a number of olfactory receptors with varying affinities. Once the odorant has bound to the odour receptor in mammals, the receptor undergoes structural changes, binds and activates the olfactory-type G-Protein, present inside the olfactory receptor neuron. The G-Protein, in turn, activates adenylate cyclase which converts ATP into cyclic AMP (cAMP). The

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cAMP opens cyclic nucleotide gated ion channels which allow Ca^{2+} and Na^{+} ions to enter the cell, depolarizing the olfactory receptor neuron and beginning an action potential which carries the information to the brain.

Each *Drosophila* olfactory sensory neuron (OSN) expresses two odorant receptors: a divergent member of the OR family and the highly conserved, broadly expressed receptor OR83b. OR83b is essential for olfaction *in vivo* and enhances OR function *in vitro*; but the molecular mechanism through which it operates is unknown. Unexpectedly, unlike all known vertebrate and nematode chemosensory receptors, it was found that *Drosophila* ORs and OR83b adopt a novel membrane topology with their N-termini and the most conserved loops in the cytoplasm (Benton *et al.* 2006). Electrophysiological analysis recently carried out provided strong evidence supporting the idea that insect ORs are in fact ligand gated non-specific cation channels. The different subunits of the OR/OR83b complex in the insect olfactory system are able to shift ion selectivity to a measurable amount, a property directly related to ion channels (Nichols *et al.* 2011). This suggests that insect ORs themselves are necessary and sufficient to produce odour-evoked responses, unlike mammalian ORs.

The structure of ORs in mammals and nematodes is characterized by seven hydrophobic, membrane spanning domains. Based on the primary sequence, GPCRs are categorized into three classes: A, B and C. According to this classification, due to their domain organization, the ORs are classified as 'GPCR class A rhodopsin like' (Jacoby *et al.* 2006). ORs have an average length of about 320 ± 25 amino acid residues. The N-terminal region in mammals, which is extracellular, contains the well conserved NXS/T consensus sequence for N-linked glycosylation. ORs in mammals are distinguishable from other GPCRs by several conserved motifs which include the LHTPMY motif within the first intracellular loop, the MAYDRYVAIC motif at the end of transmembrane helix 3 (TM3), the FSTCSSH motif at the beginning of TM6 and the PMLNPF motif in TM7. There are seven cysteine residues which are well conserved. Two of them are common to all GPCRs, while the rest are unique to ORs. The cysteines are thought to maintain the structural integrity of the proteins (Fleischer *et al.* 2009). Experimental data suggest that TM3, TM5 and TM6 are essential for odorant binding (Katada 2005).

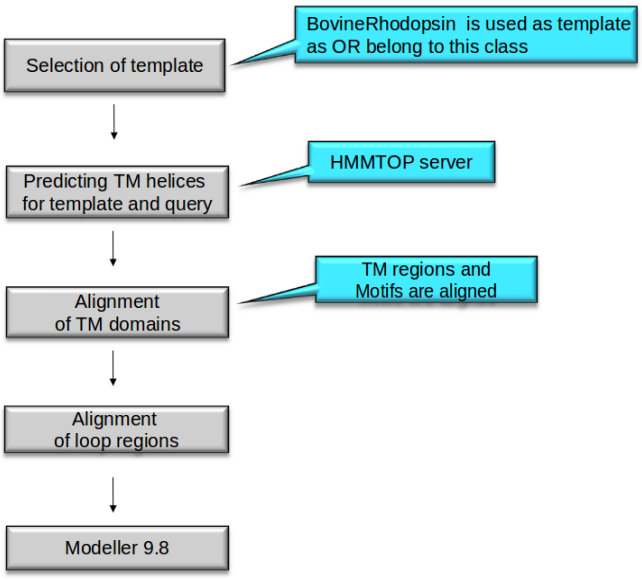

Figure 1. Flowchart depicting the steps involved in the three dimensional modelling of olfactory receptors.

Chemoreceptor gene families in *Caenorhabditis sp.* are large and evolutionarily dynamic as a result of gene duplication and gene loss. Individual mammalian and insect olfactory neurons express only one functional odorant OR. By contrast, *C. elegans* expresses multiple ORs and multiple Gα subunits on each olfactory neuron (Mombaerts *et al.* 1999).

STE2 and STE3 (α-factor mating receptors) encode GPCRs presented in the plasma membranes of haploid yeast cells (Bardwell 2004, Slessareva & Dohlman 2006). Like all other GPCRs, activation of these receptors by agonists leads to well-characterized G protein-mediated mitogen activated protein kinase signal transduction events.

Based on phylogenetic analysis, mammalian ORs can be classified into two different groups: Class I and Class II (Freitag *et al.* 1995). This classification is based on the original finding that the frog (*Xenopus sp*) genome has two different groups of ORs: one (Class I) that is similar to fish ORs and the second (Class II) similar to mammalian ORs. The comparison of structural features revealed that these two classes differ mainly in the sequence of the second extracellular loop. In humans and mouse, the majority of ORs belong to class II, but class I ORs are also found. Humans have ~ 800 OR genes, but 50% of them are psuedogenes (Freitag *et al.* 1998). Similarly, the mouse has ~2,000 OR genes, but more than 50% are psuedogenes. Out of these, 371 human ORs and 338 mouse ORs have been reported to be functional.

The recent availability of crystallographic models of a few GPCRs permits us to analyze the OR amino acid patterns in a structural context. Molecular modelling has an important role in rational drug design (Jorgensen 2004, Richon 2008) and analysis of variable structural regions. Reliable three-dimensional models from different OR phylogenetic clades can provide valuable information for structurally and functionally conserved residues. Until now, the GPCR ho-

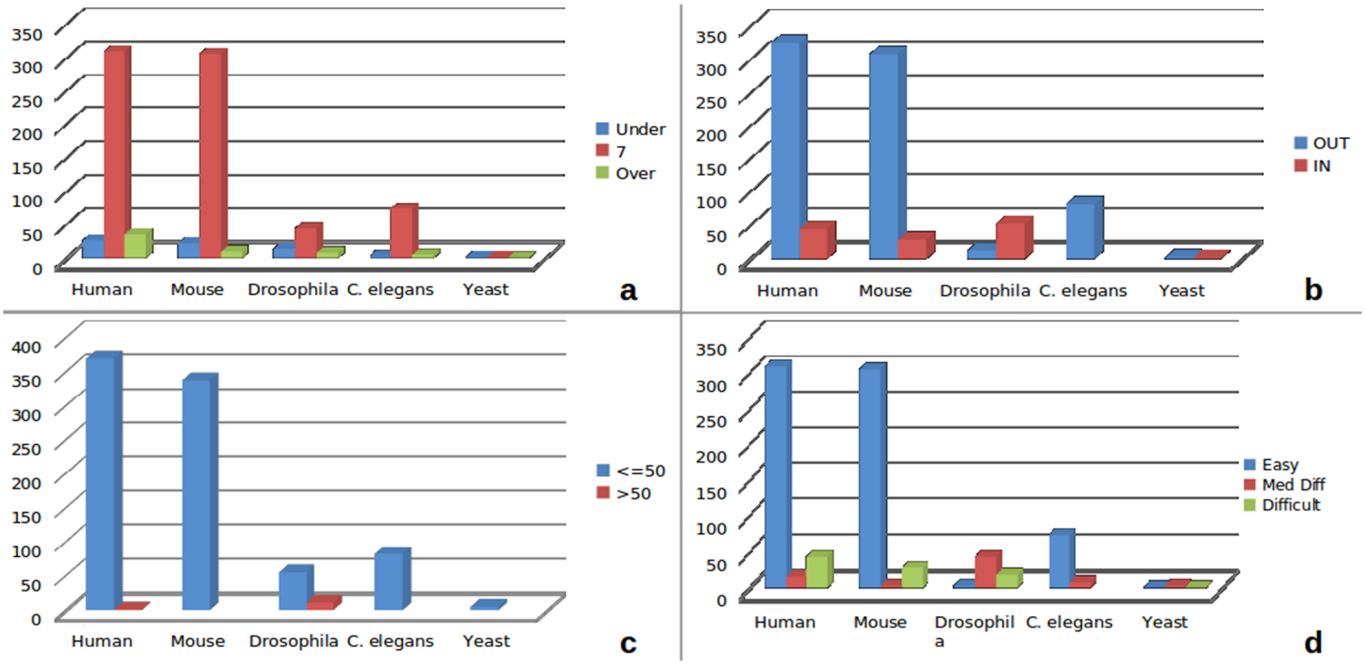

Figure 2. The number of ORs from each organism classified on the basis of (a) number of helices, (b) topology, (c) difference in loop lengths with that of rhodopsin, and (d) the number of Easy, Medium Difficult and Difficult models according to the composite classification criterion.

mology modelling methods have been based on the template of bovine rhodopsin (PDB ID: 1F88) with refinement in models achieved through energy minimizations and molecular dynamics simulations.

Structurally and functionally conserved residues have been studied and mapped on the OR models generated using homology modelling. Using the ConSurf server, functionally and structurally conserved residues have been found for the human and mouse olfactory receptors. Extracellular functionally conserved residues might be those which are involved in ligand binding, while functionally conserved membrane embedded residues might be those involved in oligomerization.

Methodology

Sequence Analysis

From the phylogenetic analysis of ORs in different organisms (Nagarathnam B, Harini K, Archunan K & Sowdhamini R, unpublished results), we collected 371 human, 338 mouse, 60 Drosophila, 84 C. elegans and 5 yeast OR sequences. The mammalian (H. sapiens and M. musculus) OR sequences were clustered into 10 distinct subclusters each. Thus, representatives were collected from every subcluster to obtain a good representation of varied OR sequences, while for *D. Melanogaster*, *C. elegans* and *S. cerivisiae*, OR sequences were collected at random. TM helices and topology (N-terminus OUT/IN) of each sequence was predicted using the transmembrane prediction server

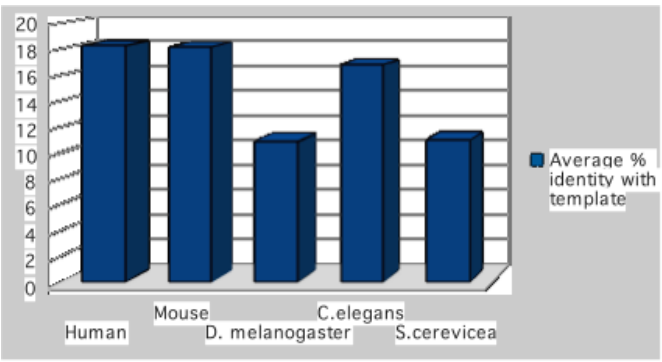

Figure 3. Average % identity of the OR protein sequences with the template (bovine rhodopsin).

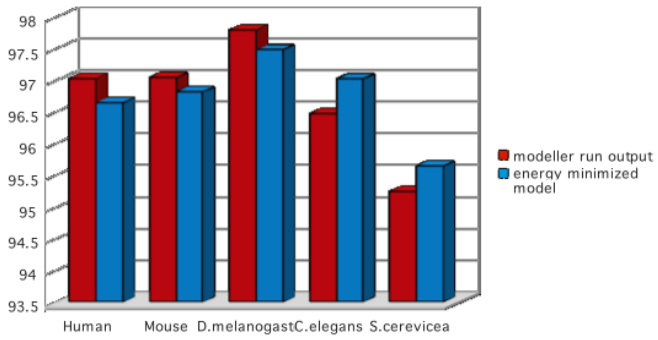

Figure 4. Procheck results for the olfactory receptor homology models.

HMMTOP (Tusndy & Simon, 2001). A composite classification scheme was employed to select representative ORs for modelling. Sequences predicted by HMMTOP server to have seven TM helices, a predicted N-OUT topology and did not differ in loop lengths from that of bovine rhodopsin (PDB ID: 1F88) by more than 50 residues were classified as 'Easy' for further homology modelling studies. The ORs which satisfied any two of the above three criteria were considered to be 'Medium Difficult'; and the rest were classified as 'Difficult'.

Molecular Modelling and Validation

The crystal structure of bovine rhodopsin (Palczewski *et al.* 2000) (PDB ID: 1F88 chain A) was used as a template for modelling all ORs. The ORs and the template were aligned using the PRALINE-TM server (Pirovano 2008) and manually edited using Jalview (Waterhouse 2009) Version 2.4. The TM region predictions from HMMTOP and the two conserved motifs MAYDRYVAIC and NPXXY in mammals were used to guide and improve the alignment of the query and the template, while no motifs were considered for the ORs from lower order organisms. The structure of the template was obtained from RCSB (http://www.rcsb.org/pdb). The coordinates corresponding to the residues 236-239 and 328-333 were not available in the crystal structure of 1F88 (chain A) due to poor electron density and hence these residues were removed from the template sequence before the alignment. The final alignment was used to construct the model using MODELLER (Sali & Blundell 1993), version 9.7 (Figure 1). A set of 20 structures were generated, from which the five least probability density function models were validated using the PROCHECK server (Laskowski *et al.* 1993). Root mean square deviation (RMSD) values between queries and template were checked using the FATCAT server (Ye & Godzik 2003), to examine if the positions of the TM helices deviate from the template after the inclusion of loop regions.

The best structure was energy minimized using the SYBYL software package version 7.2 (Tripos Associates Inc.) employing the Tripos force field, by 100 iterations of Powell's gradient with a distance dependent dielectric constant of 1 and a non-bonded interaction cut off value of 8; and was terminated at a convergence of 0.05 kcal mol^{-1}. Any residues that had been removed from the loop regions of the query to improve the alignment were added using the pre-installed PRODAT database tool in SYBYL and the structure was then energy minimized using the same parameters successively after the construction of every loop region. The final structures were further validated using the PROCHECK and FATCAT servers.

Conserved Residue analysis for human OR sequences

Representative human and mouse OR sequences were obtained from 10 clusters constructed from prelimi-

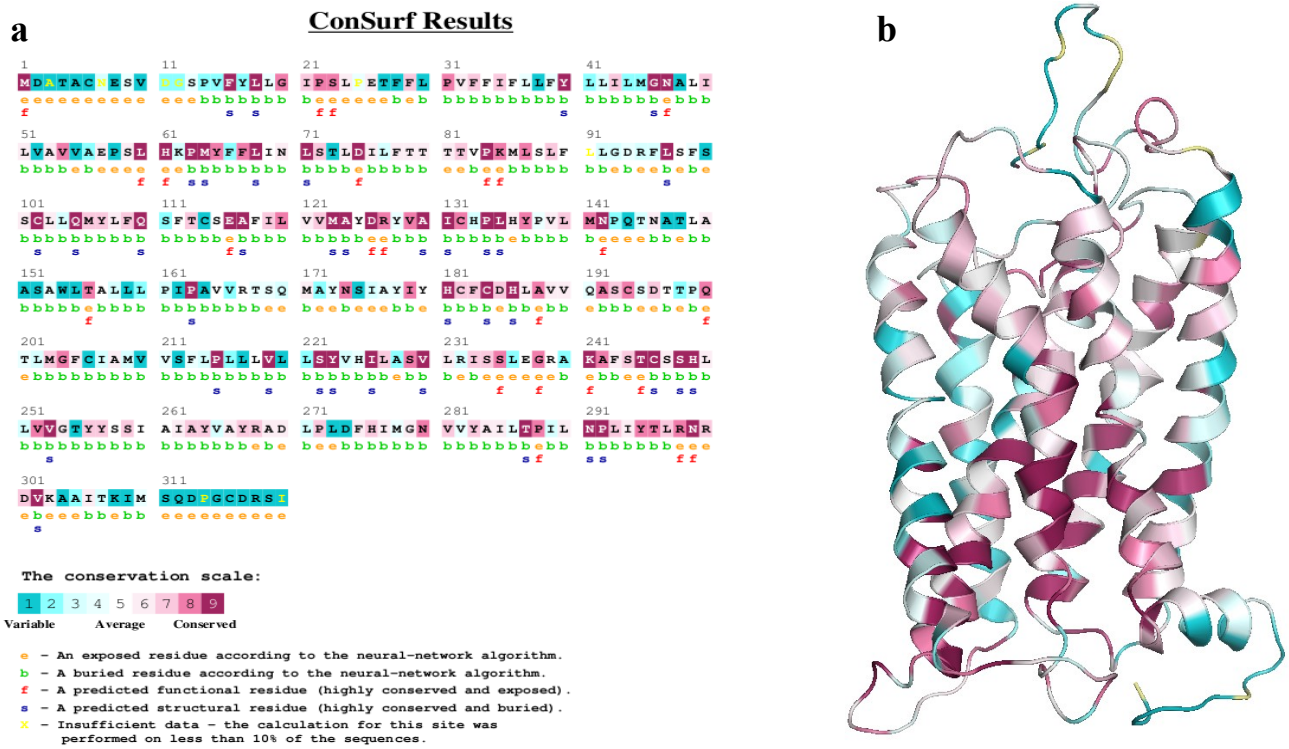

Figure 5. ConSurf result for human olfactory receptor Cluster 1 mapped on (a) sequence and (b) structural model.

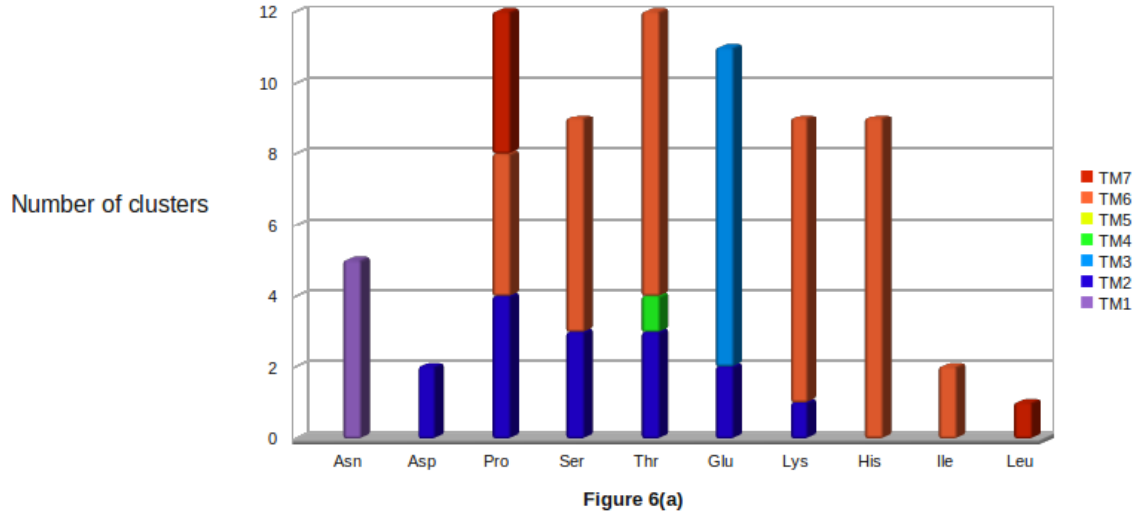

Figure 6(a)

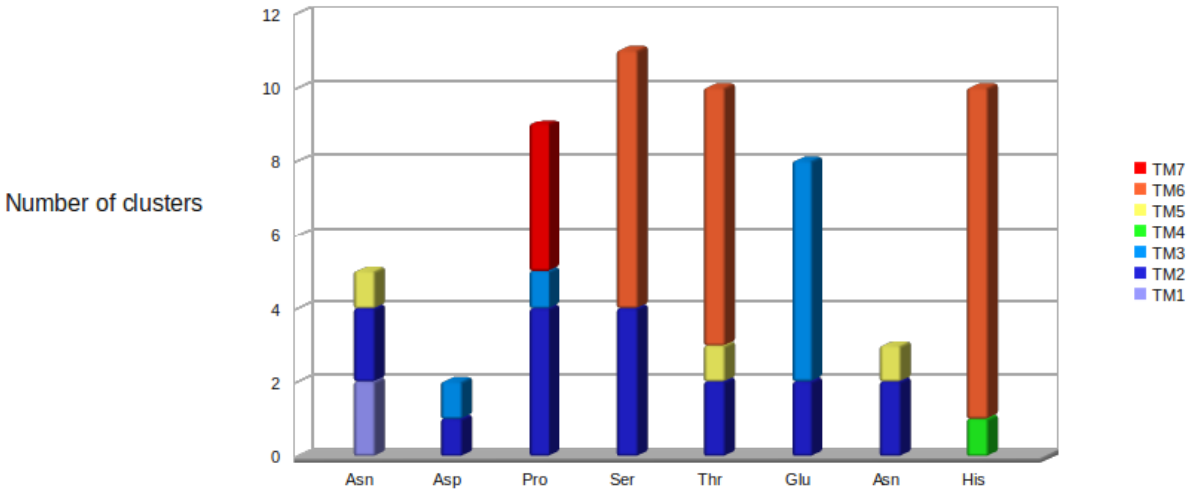

Figure 6(b)

Figure 6. (a) Conserved residues in the transmembrane regions (TM) of human olfactory receptors predicted using Consurf. (b) Conserved residues in the transmembrane regions (TM) of mouse olfactory receptors predicted using Consurf.

nary phylogenetic analysis (unpublished data). For each cluster, conserved residue analysis was done using the ConSurf server (Glaser *et al.* 2003, Landau *et al.* 2005). More specifically, the multiple sequence alignment and one representative sequence (the one selected for modelling) was given as input. The output retained the conserved residues mapped on the representative sequence and structure for each cluster. It was then analysed to check if there were any similarities in the conserved residues among the 10 clusters of the human and mouse OR phylogenetic tree, respectively.

TM-motif analysis

TM-motif (Nagarathnam *et al.* 2011) is a tool developed for annotating the positions of seven predicted transmembrane helices and conserved motifs within the aligned set of GPCR/OR protein sequences. The residues found to be conserved for representative ORs (Section 2.3) were further checked for conservation patterns using the TM-motif tool.

Dimer interface prediction for olfactory receptor structures

Dimer interfaces of ORs from *Homo sapiens*, *Mus Musculus* and *Drosophila melanogaster* were predicted by the method coded in G-protein coupled Receptor Interaction Partners (GRIP), which requires a three-dimensional structure of a target GPCR and its homologous sequences (Nemoto & Toh 2005, 2009). In this work, a model structure of a target OR and the sequences that belong to the same subtype were used. GRIP exploits three assumptions. Firstly, GPCRs form oligomers based on the domain-contact mechanism,

which utilizes the lipid-facing molecular surfaces along TM helices as the interfaces (Nemoto & Toh, 2006). Therefore, GRIP does not take into account the domain-swapping mechanism, which utilizes buried residues of a monomeric structure after the drastic conformational change of the structure (Gouldson *et al.* 2000). Secondly, the residues directly involved in the oligomerization are conserved within the subtype, to which the target belongs. Thirdly, the conserved residues would be more abundant at the interface as compared to the non-interface surface (Nemoto & Toh, 2005). Based on these assumptions, GRIP searches for the lipid-facing surfaces along transmembrane helices where a number of conserved residues are clustered with statistical significance. However, in this case, it was difficult to detect a cluster of conserved residues on the surface of the three-dimensional structure. This is because, as observed in oligomeric model structures of mouse rhodopsin (Fotiadis *et al.* 2003, Nemoto & Toh 2005), conserved residues are often observed separately at the extracellular and intracellular side along the transmembrane helices. As a result, GRIP transformed the structure as described below.

The monomeric structure of OR can be regarded as a thick tube, whose long axis is approximately perpendicular to the membrane plane. In this schematic image, all of the OR residues are regarded as constituents of the tube, and the interface residues are considered to cluster on a surface of the tube. If all of the residues are projected on the plane perpendicular to the long axis of the tube, then the projected residues form a ring-like distribution. In this regard, the interface residues would be clustered in a sector of the ring-like distribution. GRIP applied the principal component analysis to the cartesian coordinates of the alpha carbons of OR. The first principal component vector runs along the long axis of the tube-like component of the structure. Therefore, GRIP projected all the residues on the plane defined by the second and third principal component vectors, and searched for a sector where the abundance of conserved residues in the ring-like distribution was statistically significant. The residues within the sector thus detected are considered to correspond to the residues constituting the interface. In order to predict more than one interface, we removed the predicted interface residues from the data set of surface residues. Using the remaining residues, a second prediction was performed. The three-dimensional models of Homo sapiens and Mus musculus ORs were anlaysed for similarities and differences at the predicted dimer interfaces across the clusters in the respective phylogenetic tree of OR sequences.

Results and Discussion

Sequence Analysis

The sequences of the olfactory receptor proteins were classified according to the scheme described in the methodology. The results for classification of the ORs as per each criterion (number of helices, topology and loop length) and the final classes ('Easy', 'Medium Difficult' and 'Difficult') are shown in Figure 2.

Molecular modelling and validation

The bovine rhodopsin crystal structure was used as template to model the final selected ORs (Table 1S). The average sequence identity between the OR protein sequences from different organisms and the template is shown in Figure 3. Every selected OR sequence was aligned to the template independently, for homology modelling. The sequence alignment was analysed to identify conserved motifs (such as DRY at the C-terminal end of TM3 and NPXXY at TM7 in the case of mammalian ORs). Initially, the correct alignment of the TM helices of the query and template was given importance and, subsequently, the loop segments were

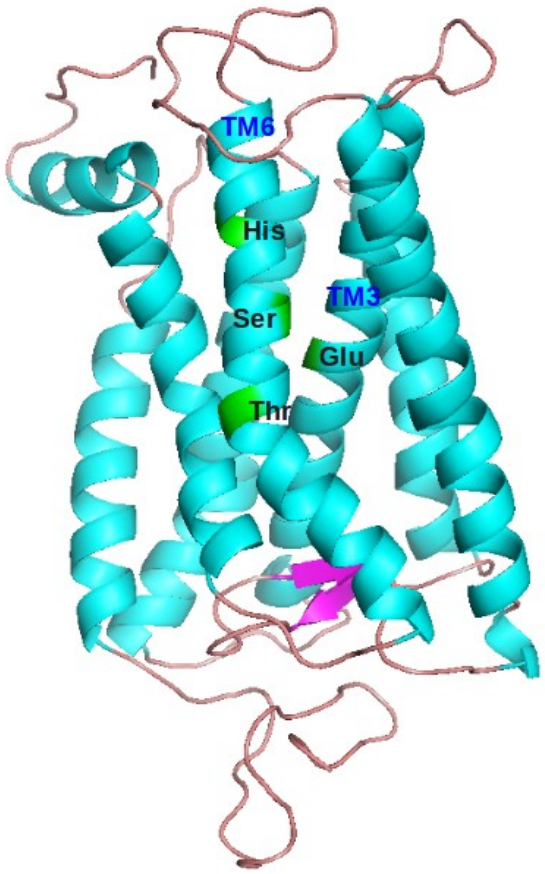

Figure 7. The highly conserved residues mapped on the human olfactory receptor model.

aligned between the two. Predicted TM helices of ORs were found to be shorter than that of the template.

The model structure after loop building and energy minimization through SYBYL was validated using PROCHECK (Laskowski *et al.* 1993). PROCHECK results for the OR protein models, excluding the loop regions, show more than 95% of residues in allowed regions (including strictly allowed and partially allowed) of the Ramachandran plot. The full-length structures for the models show more than 90% in the allowed regions (including strictly allowed and partially allowed regions) on the Ramachandran plot (Figure 4). The residues found in the disallowed regions were mainly in the loop regions which are highly variable in length and sequence identity. The RMSD of the full length OR energy-minimised models with respect to the template was found to be less than 2 Å (except in the case of yeast OR proteins where it was ~ 2.4 Å).

Modelling of the olfactory receptor protein loops proved to be a difficult task as bovine rhodopsin (the template) contains longer helices and shorter loops when compared to the predicted helices and loops of the OR sequences. Other available GPCR structures in the PDB differ only in the loop regions (length and shape). Thus, a multi-template modelling approach, using distantly related GPCR templates, would not change the MODELLER output to a great extent. In this regard, modelling of loops using different methods can be attempted in future modelling studies. In this study, predicted loops with more than 30 residues have not been considered. Since the loops themselves are not well characterized, modelling disulphide bonds as present in the template GPCR led to severe deformation of the helices. This might be because, unlike bovine rhodopsin which has one conserved cysteine in the extracellular loop 2, there are three conserved cysteines in the extracellular loop 2 of the OR sequences with no definite clue on disulphide bridge connectivity. Hence, modelling of disulphide bridges for ORs was not attempted.

A few OR sequences are predicted to have eight or six TM helices by the HMMTOP server. In the latter case, the second helix is missing while aligning with the template; whereas in the former case, an extra TM helix is predicted between TM4 and TM5 in the extracellular loop. No data is available on the functional importance of such under- and over-predicted TM helices in OR sequences. Thus, the extra helix in over-predicted sequences was excised before modelling and the region not predicted as a helix in the under-predicted sequences was forcefully aligned to the second helix of the template while modelling. Since transmembrane topology prediction does not solely depend on the number of helices, different servers could be used in the future for further analysis of such sequences. TM helices in OR which contain a Gly, Val or Pro residue within a predicted TM helix seem to unwind the helical conformation during energy minimisation and further refinement of these regions needs to be done using different energy minimization techniques and/or MD simulations.

Conserved Residue analysis

For every cluster of human and mouse olfactory receptor sequences, conserved residue analysis was performed using the ConSurf server. For a given cluster, the multiple sequence alignment and one representative sequence (the one selected for modelling) was given as input. The output was the record of conserved residues mapped on the representative sequence and structure for a given cluster (one example is shown in Figure 5a,b). The output was then analysed to check if there were any similarities among the 10 subclusters of the human OR phylogenetic tree. Residues that were predicted to be functionally important and which are conserved among all 10 clusters could possibly be general ligand binding sites across all OR structures.

A glutamate residue in TM3 was found to be conserved in 8 out of 10 human OR clusters and 6 out of 10 mouse OR clusters, while a lysine residue in TM6 is found to be conserved only in human ORs and not in mouse OR sequences. Serine, threonine and histidine residues in TM6 were found to be conserved in both human and mouse OR clusters. A few polar residues were found to be conserved in extracellular loops 1, 2 and 3, but similar conservation was not observed across all clusters indicating high variability among the ligand binding regions (Tables 2S and 3S). This was expected due to the high variability among odorant binding in ORs (Figure 6a, b). These residues are then marked on the models generated using homology modelling techniques (Figure 7).

TM-motif analysis

The residues found to be conserved, as described in Section 2.3, were further analysed to check for conservation using the TM-motif tool. The glutamate residue in TM3 was found to be conserved in 8 out of 10 clusters and replaced by glutamine in one of the clusters. Lysine, serine, histidine and threonine residues found in TM6 were also found to be conserved. Figure 8 shows the conservation of the above mentioned residues mapped on the TM-motif alignment for subcluster 1 of the human OR sequences.

Dimer-interface prediction for OR models

Interfaces of olfactory receptors were predicted by the

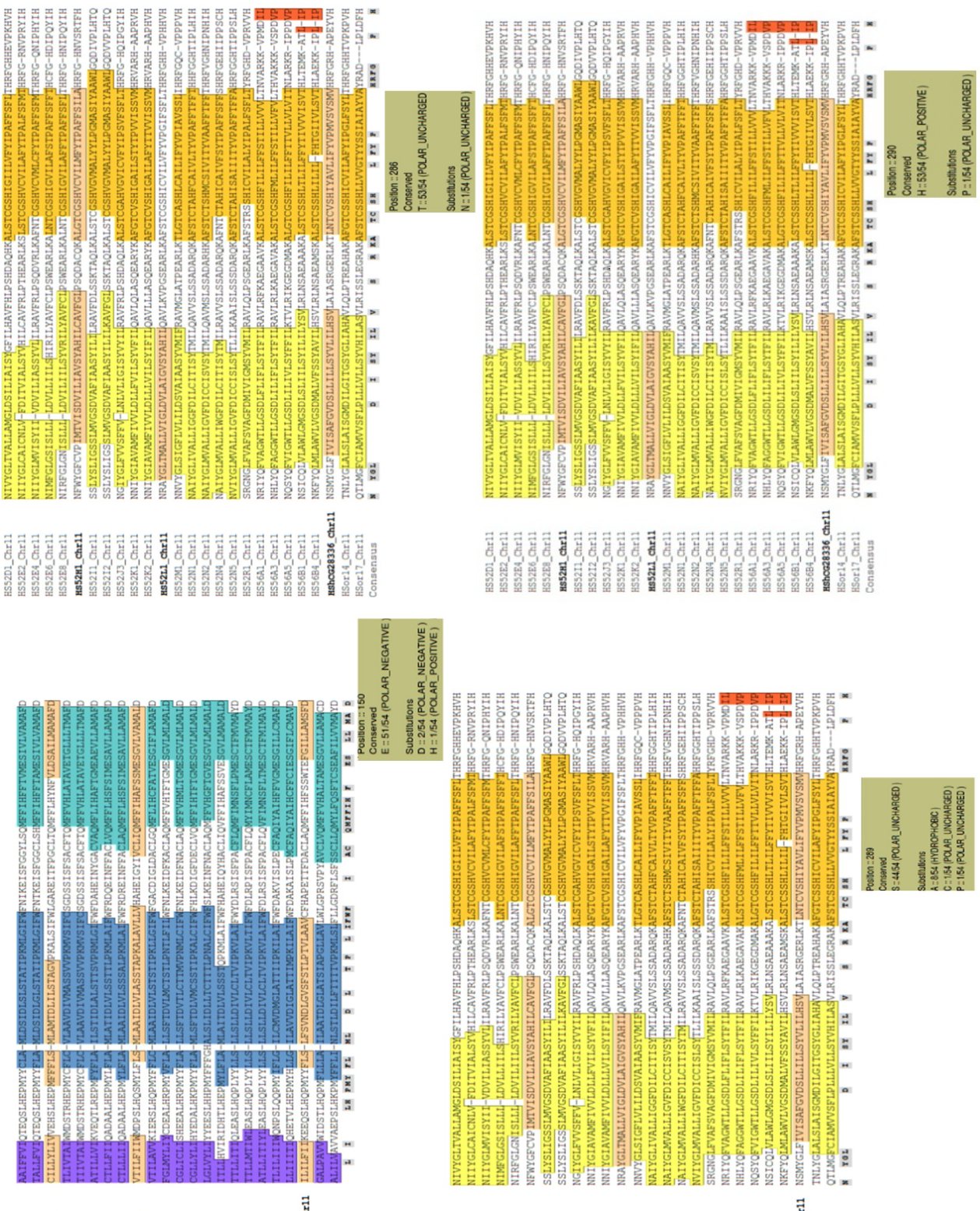

Figure 8. Conserved residues predicted in TM helices of OR marked using the TM-Motif tool. TM-motif output for Human OR cluster 1 showing conservation of the (a) Glutamate (E) residue in TM3 and (b) Threonine, (c) Serine (S) and (d) Histidine (H) residues in TM6.

method provided in G-protein coupled Receptor Interaction Partners (GRIP) (Nemoto & Toh 2005, 2009), which requires a three-dimensional structure of a target GPCR and its homologous sequences, as mentioned in the methodology. For every OR model, the primary and secondary dimer interfaces are predicted and mapped on the model. The mouse OR cluster 1 had only one sequence in our dataset and thus we were unable to predict the dimer interface for this cluster. We then checked whether there is any relation between the dimer-interface prediction and the clustering of OR sequences in the phylogenetic tree of Homo sapiens and Mus musculus. We could find that the dimer-interface prediction varies within and across different clusters of OR phylogeny (Tables 4S and 5S). These results show that the dimer-interface is highly variable and needs not be similar for highly homologous sequences (Figure 1S).

Conclusion

Olfactory receptors have in general been a very challenging and interesting system to model and analyse. The combinatorial response to odours by these receptors gives rise to hoards of data to analyse. Such analyses will in turn help us to better understand the olfactory coding. Studying a few model organisms will greatly improve our understanding of this system, as the basis for olfaction seems to be similar across different organisms. In this study, we have generated three-dimensional models for 100 ORs by homology modelling, using bovine rhodopsin as a template. These include receptors from *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*. The receptors from *Homo sapiens* and *Mus musculus* were found to form 10 subclusters, respectively, in the phylogenetic tree. In order to analyse the amino acid conservation of these 10 subclusters, we used the Consurf server to identify functionally important residues and map them on the structures generated. The residues that bind to G -proteins are found in OR sequences and are similar to known GPCR motifs. Thus, when searching for ligand binding functional residues we do not consider the above as functionally important ones. The residues in TM3 and TM6 were found to be functionally conserved, amidst other structurally conserved ones. In several earlier studies on OR sequences, the residues in the TM3-TM6 regions have been found to be conserved and involved in ligand binding (Gottlieb *et al.* 2009, Pilpel & Doron 1999). These residues were marked on the structure and on the alignment from the TM-motif tool. Dimer interface predictions were performed for the OR models to understand the oligomeric state of these receptors and the functional significance of such higher order entities. We can use these known functionally important residues as a starting point for both small-molecule and protein-protein docking studies. In the future, we plan to include such a conserved residue analysis on OR structures from other genomes and develop a database to map alignment, phylogenetic tree, residue conservation and dimer interface of OR sequences.

Conflicts of Interest

There are no conflicts of interests to declare.

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