Machine Learning-based Modeling of Olfactory Receptors: Human OR51E2 as a Case Study

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Abstract

Atomistic-level investigation of olfactory receptors (ORs) is a challenging task due to the experimental/computational difficulties in the structural determination/prediction for members of this family of G-protein coupled receptors. Here we have developed a protocol that performs a series of molecular dynamics simulations from a set of structures predicted *de novo* by recent machine learning algorithms and apply it to a well-studied receptor, the human OR51E2. Our study demonstrates the need for extensive simulations to refine and validate such models. Furthermore, we demonstrate the need for the sodium ion at a binding site near $D^{2.50}$ and $E^{3.39}$ to stabilize the inactive state of the receptor. Considering the conservation of these two acidic residues across human ORs, we surmise this requirement also applies to the other ∼400 members of this family.

Olfactory receptors (ORs) are a family of G protein-coupled receptors (GPCRs) that plays a crucial role in the sense of smell.[1] The human genome encodes for approximately

800 GPCRs, out of which 50% are ORs.[2] Although initially identified in the nose, ORs are expressed in different parts of the body.[3,4] The investigation of the physiological roles of these extranasal ORs, as well as their possible involvement in pathological conditions, is attracting a growing interest.[5,6] Moreover, given that GPCRs are the target of ∼34% of FDA-approved drugs[7] and the wide range of biologically active molecules binding to ORs,[8] these receptors are being explored as potential novel drug targets.[9,10] However, the lack of high-resolution structures for ORs has hindered the understanding of their functional mechanisms and the development of OR-targeting drugs.

Recently, the field of computational biology has made significant strides in protein structure prediction, following the development of AlphaFold2,[11] a deep learning(DL)-based algorithm that can predict the 3D structures of proteins from their amino acid sequences with high accuracy. The success of AlphaFold2 and other machine learning(ML)-based algorithms has provided a powerful tool to study protein structure and function.[12–14] Nonetheless, structural prediction of GPCRs, including ORs, still presents challenges. In particular, the algorithm predicts a single structure, despite multiple conformational states are possible for GPCRs,[15,16] and higher average confidence scores are obtained for proteins with close homologs in the training PDB set,[17] which is not the case for ORs.

To verify the reliability of an out-of-the-box *in silico* approach to predict OR structures and dynamics, we tested a set of models generated with six different predictors, followed by sub-microsecond molecular dynamics (MD) simulations. We chose to focus on the human olfactory receptor 51E2 (hOR51E2), associated to prostate cancer, because it has been widely studied, both experimentally and computationally (using a homology model).[18] Based on our test case, we propose a protocol to build reliable models of inactive, sodium-bound OR structures.

Structure prediction. A set of six structural models of hOR51E2 was generated via homology modeling and ML-based prediction algorithms. For homology modeling, we re-

lied on the SwissModel (SM) webserver,[19] while for ML-based prediction, we considered AlphaFold (AF),[11,20] RoseTTAFold (RF),[21] OmegaFold (OF),[22] and ESMFold (EF).[23] As a last candidate, we considered a model of the receptor in its inactive state (AF_{in}), generated with AlphaFold-MultiState.[15,24] For all the predictors considered, we tried to use the models already available to the public (*i.e.,* without directly using the ML algorithm or modifying the default parameters – see details in the Supporting Information).

Models without Sodium ion in its Binding Site. For the first set of MD runs, we submitted the starting configurations (solvated and embedded in a POPC lipid bilayer) as set up by the CHARMM-GUI[25] webserver (see the Methods section and the Supporting Information). During the equilibration, while the receptor and the membrane configurations were maintained in the presence of restraints, when the system was left unconstrained we observed in all cases at least a partial rearrangement of the transmembrane helices and their interfaces.

Interestingly, even before removal of the restraints on the protein structure, the interior of the receptor is flooded with water molecules passing from the intracellular to the extracellular part (Figure 1). During the last 500 ns of unconstrained simulation, the amount of flowing water increases, destabilizing the interaction network that keeps TM6 and TM7 close together, thus increasing the spacing (from $7-9$ Å to $13-15$ Å) between them and finally breaking the helical bundle fold.

One notable exception was represented by the SM structure. After ∼200 ns of unrestrained simulation, a sodium ion bound to the receptor, occupying the known ion binding pocket in class A GPCRs, close to $D^{2.50}$ (D69 in hOR51E2). In addition, $E110^{3.39}$ also participated in the coordination of the the Na^+ ion. After this event, the structure appeared much more stable (despite the already broken fold). This suggests that a sodium-bound inactive structure might be more stable than an ion-devoid configuration and thus make us consider the possibility of positioning such an ion in the Na^+ binding pocket from the beginning of the MD protocol.

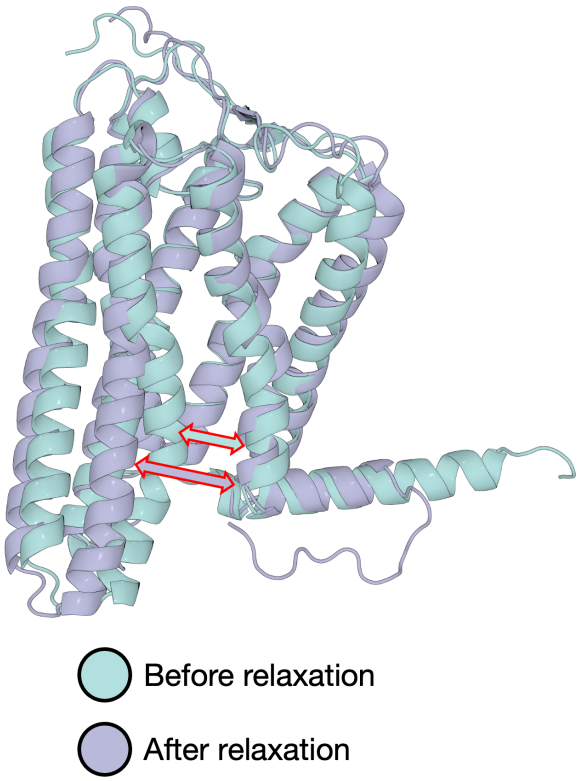

Figure 1: Opening of the TM6-TM7 interface in absence of sodium, exemplified here for the AF model. In all the simulations without a sodium ion bound to the receptor, the interface between TM6 and TM7 is disrupted (red-contoured arrows) and thus the receptor changes between a closed conformation (cyan) at the end of the restrained equilibration to a completely open conformation (lilac) after unrestrained MD.

Models with Sodium in its Binding Site. In the second set of runs, we followed the same protocol but positioning a sodium ion close to the ion binding pocket in the vicinity of $D69^{2.50}$. In all the 18 simulations (6 systems $\times$ 3 replicas per system), we observed a better preservation of the initial fold, with an RMSD of the heavy atoms around 5 Å (see Figure S2 in the SI), and the inactive conformation is maintained, as shown by the A^{100} descriptor[26] (see Figure S1). Despite this qualitative change in the stability of the fold compared to the simulations without bound Na^+, the sodium-bound simulations started from EF, RF, and SM configurations still showed, in all replicas, water passing from the intracellular to the extracellular part through the receptor, resulting in disruption of the interface between the transmembrane helices, mainly stabilized by hydrophobic interactions.

Considering the OF and AF models, water did not pass from the intracellular part to the transmembrane part of the receptor in one and two replicas out of three, respectively, maintaining the initial fold and the TM6-TM7 distance through the whole 500 ns simulations. Finally, for AF_{in} all the 3 runs maintained the original configuration (the complete list can be found in Table S2 in the Supporting Information).

To highlight differences and similarities in the fold suggested by different structure predictors, we performed a cluster analysis of the simulations. In particular, we concatenated all the MD trajectories and calculated the reciprocal RMSD of all the frames (Figure 2), considering the heavy atoms of the transmembrane helices only and ignoring the extra- and intracellular loops, which are less stable and usually predicted with a smaller confidence.[27–29] Then, clustering was performed with the gromos method,[30] as implemented in GROMACS, using an RMSD cutoff of 2.5 Å. From the cluster analysis we can make two observations: (i)

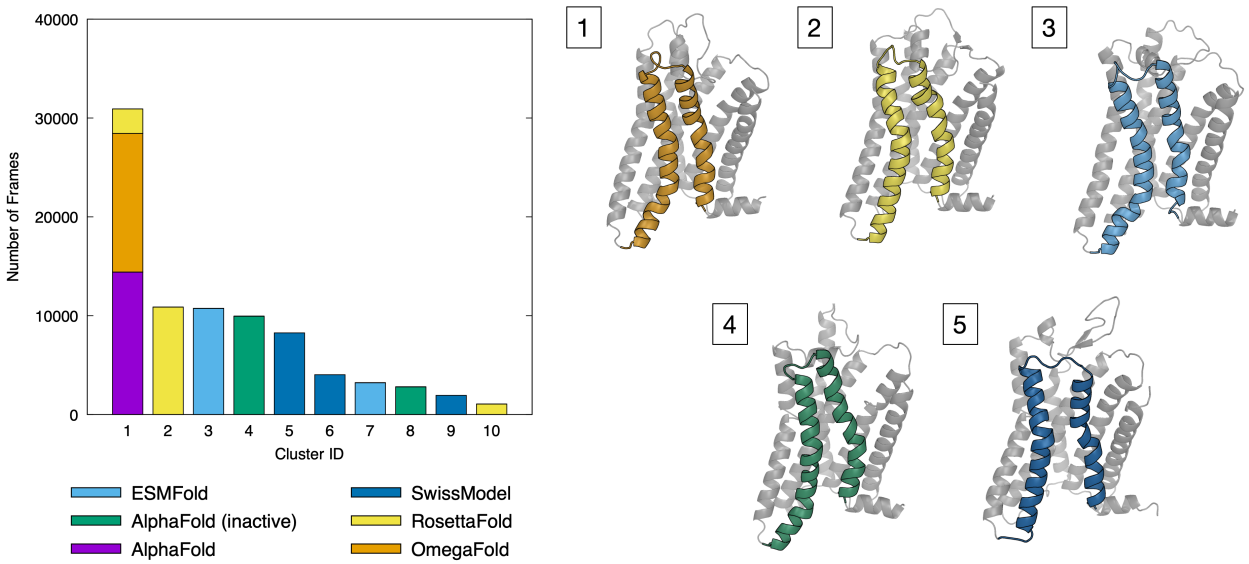

Figure 2: Cluster analysis for the sodium bound simulations started from different initial receptor configurations. *Left*, cluster population; right, representative structures of clusters 1-5, with TM6 and TM7 helices colored according to the starting model.

the three most stable models –AF_{in}, AF, and OF– belong to two different clusters (1 and 4 in Figure 2); (ii) the histogram shows no overlap between the different source structures (with the exception of cluster 1, where part of RF and whole AF and OF trajectories are classified

together). Therefore, upon refinement with MD, the different structure prediction methods return significantly different conformations in the transmembrane part of the receptor, even though the helical bundle should be less prone to errors in the structure prediction (and thus more stable). Interestingly, RF (which unfolds during the simulation) overlaps, at least in part, with the conformations sampled in the AF and OF simulations (see cluster 1 in Figure 2). In general, AF and OF seem to generate similar initial and MD-refined structures, that, considered together, are stable in three out of six simulations.

The most evident change between the three best candidates, AF_{in} and AF/OF, is the different structural alignment of the TM6-TM7 interface, as shown by the corresponding representative structures in Figure 2 (panels 1 and 2). Contact map analysis of the centroid structures of clusters 1 and 4 (using MAPIYA[31] (https://mapiya.lcbio.pl)) reveals a shift in the non-bonded (mainly hydrophobic) interactions that stabilize the TM6-TM7 interface in the two structures (see Figure 3). The TM6 sequence is half helical turn behind in the AF_{in} model with respect to AF/OF, whereas the TM7 helix is similar in both models. As a result, a mismatch between opposing amino acid pairs occurs at the TM6-TM7 interface. In partic-

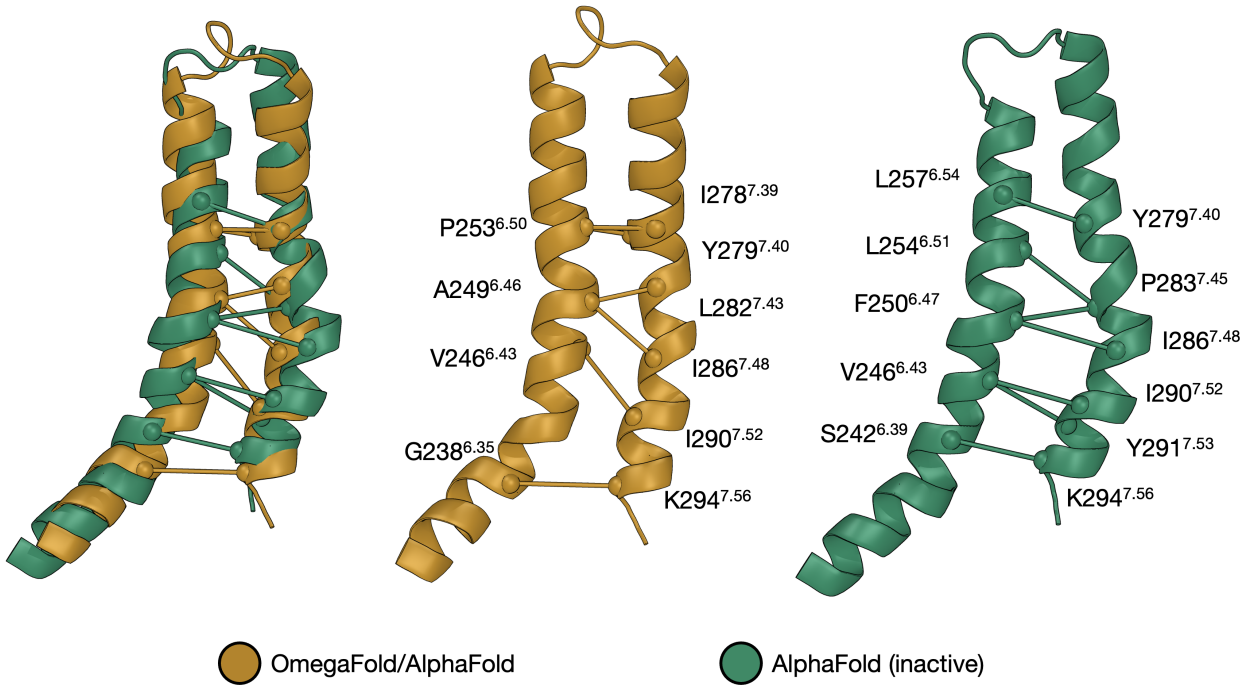

Figure 3: Comparison of the TM6-TM7 interface in OF/AF and AF_{in} models.

ular, the AF_{in} and AF/OF structures have almost the same TM7 residues involved in the interhelical contacts (Y279$^{7.40}$, I286$^{7.48}$, I290$^{7.52}$), while for TM6 the only residue identified as interacting in both models is V246$^{6.43}$. From a practical point of view, TM6 appears to be shifted by 1-2 residues in the structural alignment of the two models, similarly to what was observed in a recent work on another chemosensory receptor, TAS2R14,[32] when comparing two models built with AlphaFold and I-TASSER, respectively.[33]
This observation exemplifies that, although the global differences between structures generated with different predictors might seem minimal, small local differences can still result in significant changes and thus in misleading predictions regarding structure-function relationships.

In conclusion, we set up a protocol to equilibrate and test models of olfactory receptors in their inactive state embedded in a POPC membrane. We can highlight four main observations from the protocol: (i) as already suggested in previous works,[34–36] the reliability of structures should be tested extensively by MD simulations; (ii) *de novo* structural determination can lead to significantly different predictions in presence of a multi-state system (see AF vs. AF_{in}); (iii) the absence of conserved motifs between human ORs and other class A GPCRs, especially for TM6,[37] can lead to gross errors in structure reconstruction; and (iv) for hOR51E2 in its inactive state –but this is most probably valid for a large set of ORs– the presence of sodium in its binding pocket is crucial for the stabilization of its fold. Sodium binding to hOR51E2 can be attributed to the presence of two negatively charged residues, D69$^{2.50}$ and E110$^{3.39}$. The first one is a known site for ion binding conserved in class A GPCRs, while the second position is usually occupied by S in non-olfactory class A GPCRs.[38] Instead, 93% of human ORs contain Asp/Glu at both positions 2.50 and 3.39 (see Figure S3). Residue conservation in these sites can suggest a coevolutionary feature[39] supporting the structural stability role of Na^+ ion binding, as empirically observed by us. In line with this hypothesis, the presence of sodium in that position is

also foreseen for hOR51E2 by the ML-based protein-ligand binding predictor, AlphaFill[40] (see https://alphafill.eu/model?id=Q9H255). As a further indirect validation, a recent experimental work[41] showed that mutation of $E^{3.39}$ enhances the *in vitro* expression of ORs, further supporting the structural and functional importance of this residue. As pointed out by a recent commentary,[42] *de novo* structure determination is dramatically limited by the "single answer problem": predictors return a single structure that is, following the training, the most probable candidate. From a general point of view, this can be correct only for single-state proteins, while here (and in the majority of the biologically-relevant cases) our target GPCR has a set of different conformational states. This problem can be solved (or attenuated) taking particular care of the structural knowledge that the algorithm employs to perform its prediction. In the Heo and Feig[15] or del Alamo *et al.*[16] approaches, this is accomplished by limiting the training set to a single state (here GPCR experimental structures annotated to be in the inactive state), to maximize the chances of a correct prediction. The majority of the *de novo* structure determination algorithms need a properly aligned multiple sequence alignment (MSA). In the case of GPCRs, and in particular ORs, special care needs to be taken when generating such MSA, especially in the absence of known conserved motifs. In particular, ORs lack the "rotamer toggle switch" involving $W^{6.48}$ present on helix TM6 in non-olfactory class A GPCRs,[37] but contain Y/F at the 6.48 position.[43] Such divergence (and possible consequent MSA mismatch) may result in different structural predictions. Some of these models seem to be not good enough, as evidenced by the stability (or lack thereof) of the predicted fold of the system in MD simulations. One possible way to overcome this problem can be represented by the use of manually-curated MSA based also on structural information and/or in the training of ML weights to target specific GPCRs subfamilies in their structure predictions.

Finally, during the writing of the manuscript, a preprint was published[44] presenting the first ever human OR structure determined experimentally, and coincidentally the structure corresponds to a construct based on the hOR51E2 sequence in complex with an agonist

and a mini-Gs protein. Unfortunately, as of March 2023, the coordinates have not yet been deposited to the Protein Data Bank and thus we cannot make a direct comparison between our AF^{in} or AF/OF inactive models and the agonist-bound, active cryo-EM structure. In the future we plan to integrate this structural information to check our findings and build a better MSA for future structural predictions of olfactory receptors.

Methods

System preparation

We preprocessed all the structures obtained using the Protein Preparation Wizard implemented in Schrödinger Maestro 2022-3,[45] which automatically assigns the amino acid protonation states. Two exceptions were represented by $D69^{2.50}$ and $E110^{3.39}$, that were kept in their charged state. All the structures prepared were further processed via the interface of CHARMM-GUI.[25,46] First, we built a disulfide bond between $C96^{3.25}$ and $C178^{45.50}$, then we defined a cubic box with dimensions $100 \times 100 \times 120 Å^3$, with the receptor embedded in a POPC lipid bilayer. The membrane and the receptor were solvated in water with a NaCl concentration of 0.15 M, in line with standard experimental and physiological conditions for GPCRs. The protein, lipids, and ions were parameterized using the CHARMM36m force field,[47] while water was described with the TIP3P[48] model.

Molecular dynamics simulations

The simulations performed here were based on an extended version of the standard CHARMM-GUI workflow (see Supporting Information). The production step was a 500 ns-long unrestrained MD simulation with a time step of 2 fs. Velocity rescale thermostat[49] and cell-rescale barostat[50] were applied to keep the temperature and pressure to 310 K and 1 bar, respectively. For all Na^+-bound systems, we performed three independent replicas for each model, assigning different starting initial velocities. All simulations were performed using

GROMACS[51] 2021.2 patched with PLUMED.[52,53]

Data Availability

Data needed to reproduce the results shown in this paper (structures, topology, GROMACS and PLUMED input files, etc.) are available at Zenodo (https://doi.org/10.5281/zenodo.7708574).

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Supporting Information Available

Extensive details on the initial structures, simulation protocol steps, description of the A^{100} index for hOR51E2 and its implementation in PLUMED, figures with the time evolution of A^{100} and RMSD, a table with the water passage results, a plot that shows the amino acid conservation for positions 2.50 and 3.39, as well as a table with the Ballesteros-Weinstein numbering for hOR51E2, as listed in the GPCRdb.[24]

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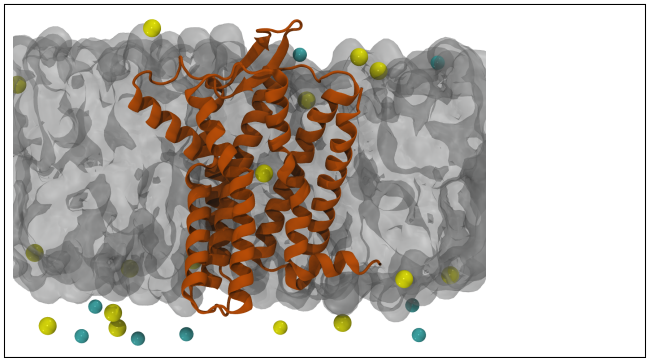
TOC Graphic