A 3D Model for Human Melanocortin 4 Receptor Refined with Molecular Dynamics Simulation

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A B S T R A C T

Despite a quite short early history, computational drug design and discovery methods can now be efficient in reducing costs and speeding up drug developing procedure. Melanocortin-4 receptor (MC4R) is a G protein-coupled receptor implicated in the regulation of body weight. Despite its clinical reputation, there is a lack of in-depth knowledge about structure and behavior of MC4R in lipid bilayer due to the absence of a crystal structure. In this context, a computational investigation was presented to study the Melnocortin 4 receptor (MC4R) receptor integrating homology modeling (HM) and molecular dynamics (MD) simulations. A homology-based model of the MC4R receptor was produced. The resulting homology model of the receptor was then used for molecular dynamics simulation studies in explicit POPC. The receptor structure that ensued was refined and the final native conformation was obtained.

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Introduction

Maintenance of body weight is controlled by complicated factors such as measurement of energy intake, external environmental conditions, and feedback that then adjusts further feed intake and expenditure. Therefore, numerous behavioral, environmental, and genetic factors can cause obesity. Different mutations in the leptin and the leptin receptor have given some useful information on genetic components involved in the regulation of energy balance in body [1]. Among various proteins, one of them with particular interest that is involved in the regulation of energy homeostasis is the melanocortin-4 receptor (MC4R). The MC4R response to leptin signaling is a link between food intake and body [2]. Melanocortin receptors belong to the superfamily of G protein-coupled receptors (GPCRs). Five melanocortin receptor subtypes have been identified thus far. They differ in their tissue distribution as well as physiological functions. MC4R is found primarily in the brain [3].

In structure-based drug design procedure, knowledge of the three dimensional (3D) structure of a target protein is of greatest importance. The 3D structure of a typical protein is either experimentally determined by X-ray crystallography or by NMR spectroscopy. However, the rate at which protein sequences are currently being introduced exceeds by far the rate at which 3D structures of target proteins are introduced experimentally. Thus, for a considerable number of protein targets, the 3D structure will not be readily available. In such cases a computational protein modeling is a suitable alternative way.

The final aim of protein modeling is to forecast a reliable structure from its sequence with an accuracy that is comparable to the best results obtained experimentally. This would permit researchers to safely apply quickly generated in silico protein models in all the fields where today only experimentally achieved structures provide a firm basis such as structure-based drug design, analysis of protein function, interactions, antigenic behavior, and rational design of proteins with increased stability or novel functions. On the other hand, the protein modeling is the only way to find structural information if experimental techniques be unsuccessful. It must be noted that many proteins are simply too large or unstable for analysis by experimental techniques such as NMR and cannot be crystallized by X-ray crystallography. Among

computational methods for modeling of protein structures, the most reliable computer-based method for developing a three-dimensional protein structure is homology modeling (HM) [4].

However, HM only considers information available from the template protein structures. The model remains at a rather approximate level if in the target protein several amino acids of the active site are replaced with respect to those in the template protein(s) [5].

The super family of G-protein coupled receptors (GPCRs) includes a large part of present drug targets (about 40 to 50 percent) [6]. These transmembrane receptors receive different extracellular signals such as photons, peptides, proteins, lipids, eicosanoids, purines, nucleotides, excitatory amino acids, ions, or small molecules such as serotonin (5-HT) and then, following a geometrical change of the protein, propagate the signal across the lipid bilayer to the intracellular space.

The recent introduction of the crystal structure of GPCR proteins such as bovine rhodopsin (Rh) (that is the first prototypical GPCR structure to be solved at high resolution) has made possible homology-based model production of other GPCRs. Typically, a crystal of GPCRs can be achieved from X-ray/neutron scattering, or a solution phase NMR structure such as those provided through the protein databank (http://www.pdb.org/). When such 3D experimental structures are not available, researchers typically can use a homology model as an initial starting structure. It is known that an ineffective refinement technique is one of the three main sources of errors affecting structures obtained from HM, together with inappropriate template selection and inaccurate [7]. One of the most important uses of molecular dynamics simulations (MDS) of proteins is actually the refinement of homology model structures. The coupling of HM techniques with MDS is helpful in that it tackles the sampling deficiency of conformational space of dynamics simulations by providing good quality initial guesses for the proteins. Indeed, HM provides the requirements of force fields to explore the huge conformational space of protein structures. The homology model of human MC4R (PDB code) has recently been released [8].The main drawbacks of this structure is not using of MD. In this study, a homology model of the MC4R is presented. This

model is employed as the starting point for nanosecond-duration MDS in a phospholipid bilayer.

Materials and Methods

Homology Modeling (HM)

In the absence of a crystal structure, the 3D structure of MC4R was predicted by HM using extensive primary and secondary structural knowledge. The primary sequence of MC4R protein was retrieved from UniProtKB/Swiss-Prot database (primary accession number: P32245) [9]. First, sequence alignments must be performed to identify conserved residues and motifs that might have structural and functional implications. The sequence of MC4R, consisting of 332 residues, was retrieved from the SWISSPROT database [10].

One of the most important challenges in HM is to find a suitable template. SWISS_MODEL was employed to find the homologous protein(s) with known structures to be used as the template in the process of MC4R 3D model building [11]. To select templates for a given protein, the sequences of the template structure library are searched.

The structure of human adenosine receptor (PDB ID: 2YDO) was selected as the template of HM procedure [12]. Several helix prediction methods were applied in a direct manner using their websites to allocate MC4R helixes including: HMMTOP [13], TOPCONS [14], OCTOPUS [15], Philius[16], TMpred[4], TMmod[17], PolyPhobius[18], split. pmfst[19], TMHMM[20], DAS[21].

The MODELLER [22] program version 9.10 was used to construct the human structure. . MODELLER is a program that models 3D of proteins and their assemblies by satisfaction of spatial restrains [23]. MODELLER tools can also be used for *de novo* modeling of loops, and model assessment [24].

From the 1000 model generated with MODELLER with the same alignment, the one corresponding to the lowest value of the probability density function (*pdf*) and fewest restraints violations was selected for further analysis. An *ab* initio algorithm implemented in the software was used to refine some of the loops of the selected model.

The backbone root mean square deviations (RMSDs) of the models relative to the templates were calculated using MODELLER. The RMSD differences from template geometry for bond lengths and bond angles were also calculated using MODELLER. The overall stereochemical quality of the final developed model was assessed by the program PROCHECK [25]. G-factor was calculated for the developed model using PROCHECK. Environment profile of final developed model was checked using Verify-3D (Structure Evaluation Server)[26].

Molecular Dynamics Simulation

Molecular dynamics (MD) simulation was carried out using the 4.5 version of GROMACS software (www.gromacs.org), using GROMOS96 forcefield[27]. The structure of MC4R obtained from the HM procedure was used as starting point for the MD simulation. Protein was soaked in a cubic box of SPC (Single Point Charge) water molecules [28] and simulated using periodic boundary conditions. All the protein atoms were at a distance equal or greater than 0.6 nm from the box edges.

MD simulation was carried in the isobaric-isothermal ensemble (NPT: 300 K and 1 bar). Using the productive MD, pressure was kept constant at 1 bar by altering the box dimensions and the time-constant for pressure coupling was set to 1 ps. The LINCS [29] algorithm was employed to constrain bond lengths, permitting the application of 2 fs time step.

The particle mesh Ewald method (PME) was used for the computation of the electrostatic forces [30]. Van der Waals and Coulomb interactions were calculated within cutoff of 10 angstrom.

Results and discussion

Homology modeling

Various steps involved in building and validation of homology model of MC4R are represented as a flow chart in Figure 1.
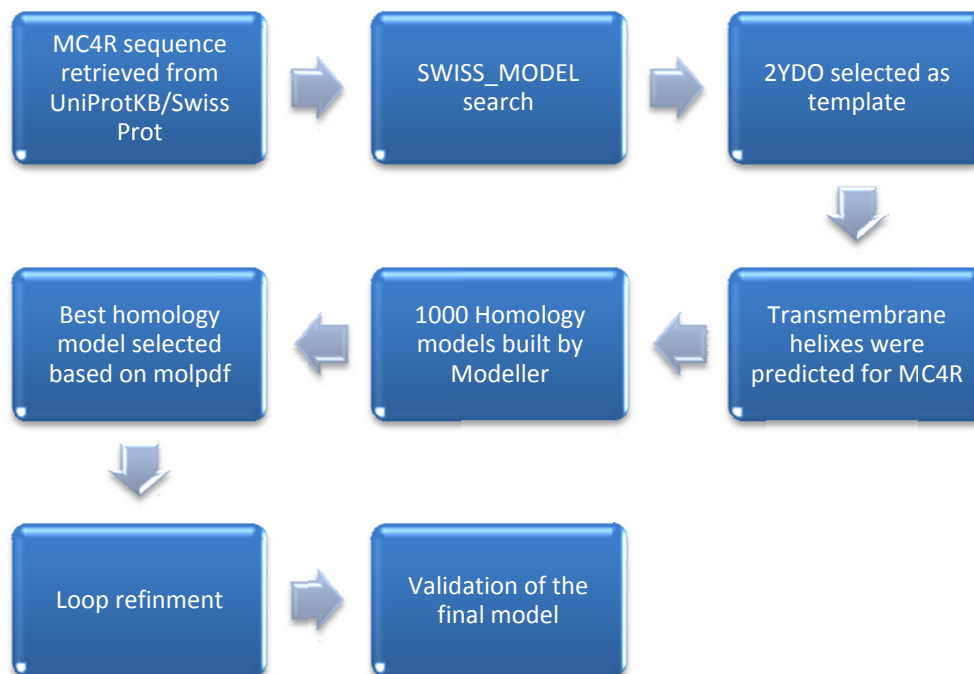
Fig. 1. Flow chart showing the scheme for HM and validation of MC4R receptor.

In the first step, we look for a previously determined structure of high "sequence identity" with the MC4R. The chain A of human adenosine receptor (PDB ID:2YDO) was founds by SWISS_MODEL analysis and hence selected as templates for developing the model.

After predicting helices by servers mentioned above, a so-called consensus approach was employed [31-33]. In this method for each residue, a consensus prediction was calculated by counting the number of servers that predicted the residue as being in a helix. For example, consensus 100% was allocated to a residue that was predicted as helix by all of the 9 servers. Table 1 summarizes the obtained prediction results of the number of helices and their locations in MC4R sequence. Consensus 8%, 16%, 25%, 50%, 75%, and 100% of applied methods which predict α-helices for the sequence are assigned and shown in Figure 2.

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                    1...|...10....|...20....|...30....|...40....|...50....|...60....|...70....|...80....|...90....|
Target sequence     MVNSTHRGMHTSLHLWNRSSYRLHSNASESLGKGYSDGGCYEQLFVSPEVFVTLGVISLLENILVIVAIAKNKNLHSPMYFFICSLAVADMLVSV
Cons.   |   8       -------------------------------------------HHHHHHHHHHHHHHHHHHHHHHHHH------HHHHHHHHHHHHHHHHHHH
Cons.   |  16       -------------------------------------------HHHHHHHHHHHHHHHHHHHHHHHHH------HHHHHHHHHHHHHHHHHHH
Cons.   |  25       -------------------------------------------HHHHHHHHHHHHHHHHHHHHHHH-------HHHHHHHHHHHHHHHHHHH
Cons.   |  50       ------------------------------------------HHHHHHHHHHHHHHHHHHHHHHH-------HHHHHHHHHHHHHHHHHHH
Cons.   |  75       ------------------------------------------HHHHHHHHHHHHHHHHHHHHH--------HHHHHHHHHHHHHHHHHHH
Cons.   |100       ------------------------------------------HHHHHHHHHHHHHHHHHHHH---------HHHHHHHHHHHHHHHHHHH
H-Score |          -----------------------------------------11129999999999999999999961------3778999999999999999
                    ..100....|..110....|..120....|..130....|..140....|..150....|..160....|..170....|..180....|..190
Target sequence     SNGSETIVITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMTVKRVGIIISCIWAACTVSGILFIIYSDS
Cons.   |   8       HHHHHHHHHHH---------------HHHHHHHHHHHHHHHHHHHH----------------HHHHHHHHHHHHHHHHHHHHHHHHHHHHH--
Cons.   |  16       HHHHHHHHHHH---------------HHHHHHHHHHHHHHHHHHHH----------------HHHHHHHHHHHHHHHHHHHHHHHHHHHHH--
Cons.   |  25       HHHHHHHH----------------HHHHHHHHHHHHHHHHHHHH----------------HHHHHHHHHHHHHHHHHHHHHHHHHHHHH--
Cons.   |  50       HHHHHHHH----------------HHHHHHHHHHHHHHHHHHHH-------------------HHHHHHHHHHHHHHHHHHHHHHHHHHHH--
Cons.   |  75       HHHHHHHH-------------------HHHHHHHHHHHHHHHHHHHH-------------------HHHHHHHHHHHHHHHHHHHHHHHHHHH---
Cons.   |100       H-------------------------HHHHHHHHHHHHHHHHHH-----------------HHHHHHHHHHHHHHHHHHHHHHHHHHHH----
H-Score |          86652222111---------------35889999999999999999999991----------------12779999999999999999999963--
                    ....|..200....|..210....|..220....|..230....|..240....|..250....|..260....|..270....|..280....|
Target sequence     SAVIICLITMFFTMLALMASLYVHMFLMARLHIKRIAVLPGTGAIRQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMSHFN
Cons.   |   8       HHHHHHHHHHHHHHHHHHHHHHHHHHHH--------------------HHHHHHHHHHHHHHHHHHHHHHHHHHHH----------HHHH
Cons.   |  16       HHHHHHHHHHHHHHHHHHHHHHHHHHHH--------------------HHHHHHHHHHHHHHHHHHHHHHHHHHHH----------HHHH
Cons.   |  25       -HHHHHHHHHHHHHHHHHHHHHHHHHH---------------------HHHHHHHHHHHHHHHHHHHHHHHHHHH-----------HHHH
Cons.   |  50       -HHHHHHHHHHHHHHHHHHHHHHHHHH---------------------HHHHHHHHHHHHHHHHHHHHHHHHHHH-------------HH
Cons.   |  75       --HHHHHHHHHHHHHHHHHHHHHHHH-------------------------HHHHHHHHHHHHHHHHHHHHHHHH--------------HH
Cons.   |100       --HHHHHHHHHHHHHHHHHHHHHH---------------------------HHHHHHHHHHHHHHHHHHHHHH----------------
H-Score |          1499999999999999999999985443111---------------------368999999999999999999887331----------2266
                    ..290....|..300....|..310....|..320....|..330..
Target sequence     LYLILIMCNSIIDPLIYALRSQELRKTFKEIICCYPLGGLCDLSSRY
Cons.   |   8       HHHHHHHHHHHHHHHHHH------------------------
Cons.   |  16       HHHHHHHHHHHHHHHHHH------------------------
Cons.   |  25       HHHHHHHHHHHHHHHHHH------------------------
Cons.   |  50       HHHHHHHHHHHHHHHHHH------------------------
Cons.   |  75       HHHHHHHHHHHHHHHHHH------------------------
Cons.   |100       HHHHHHHHHHHHHHHHHH------------------------
H-Score |          8999999999999998877
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Fig. 2. Consensus of 8%, 16%, 25%, 50%, 75% and 100% of each residue in MC4R predicted as helix by 9 different helix prediction methods. Each residue has a score from 1 to 9 based on how many methods predicted it as helix.

Table 1. Prediction of transmembrane regions of MC4R by various methodologies.

Helix	HMMTOP	TOPCONS	OCTOPUS	Philius	TMpred	TMmod	PolyPhobius	Split.pmfst	TMHMM	DAS
I	50-69	50-70	50-70	49-69	50-70	50-70	50-70	46-71	50-69	50-68
II	78-96	79-99	79-99	78-98	78-96	82-106	79-99	79-95	81-103	80-91
III	127-145	125-145	125-145	123-145	124-145	125-145	124-145	123-146	123-145	128-144
IV	166-186	168-188	168-188	165-187	166-187	166-186	166-186	164-187	166-188	166-188
V	193-212	193-213	193-213	193-216	192-213	193-217	192-217	191-220	192-214	190-217
VI	243-267	244-264	244-266	243-267	246-267	245-269	244-269	243-270	245-267	245-269
VII	286-304	284-304	284-304	282-304	286-304	284-304	284-304	278-300	282-304	283-297

To align the sequence of MC4R receptor with that of template, the CLUSTALW program was employed directly from its website athttp://www2.ebi.ac.uk/CLUSTALW[34].

The alignment was adjusted manually based on the results of TM helix prediction procedure and conserved key residues of GPCRs suggested by Baldwin et al.[35].

An optimal sequence alignment is necessary to the success of HM procedure. The sequence identity between target and template is; 30%. To optimize the alignment of different regions between template and

target, the initial alignment was adjusted so as to maximize overlap between the predicted locations of the TM helices in homology model and their locations in the pdb structure of MC4R. This provided a first-pass refinement of the alignment of the helices.

Validation of HM structure

Good overall stereochemistry is obtained for the model with 87.6% of the residue psi/phi angles falling in the most favored regions and 11.1% in the allowed region (Fig. 3).
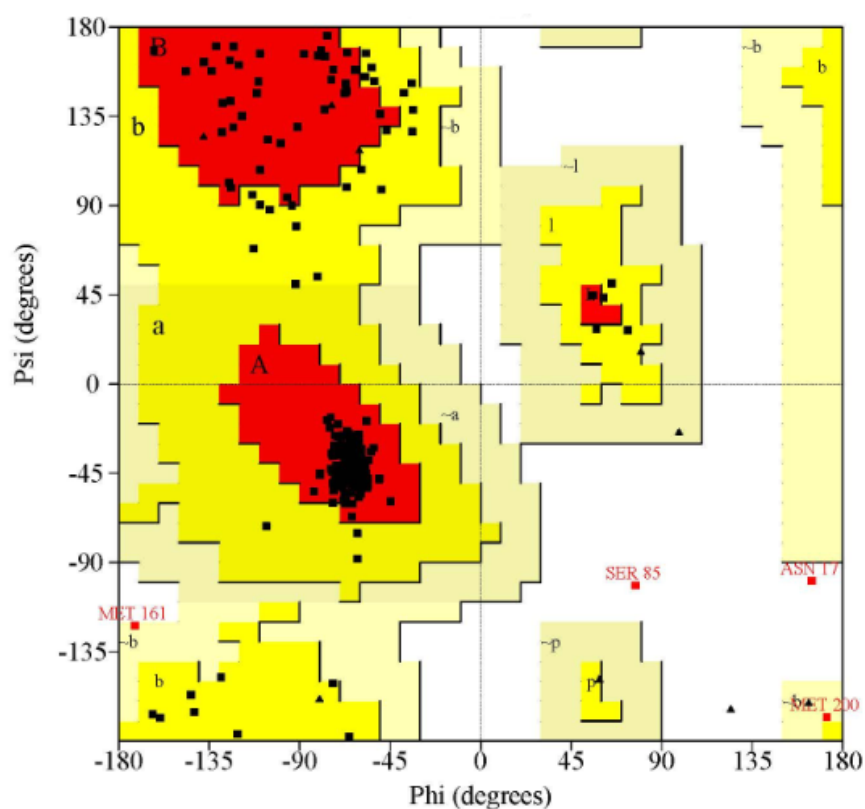

Fig. 3. Ramachandran plot of MC4R obtained from HM.

VERIFY3D is another tool proposed by Eisenberg and co-workers that derives a "3D–1D"profile based on the local environment of each residue (6). This "3D–1D"profile includes the statistical preferences

for the following criteria: (i) the area of the residue that is buried; (ii) the fraction of side-chain area that is covered by polar atoms (oxygen and nitrogen); (iii) the local secondary structure (Table 2).

Table 2. Quality of MC4R in HM and MD checked by PROCHECK and ERRAT

	Region%				G-factor		Overal quality factor of ERRAT
MC4R	Favored	Additional allowed	Generously allowed	Disallowed	Dihedrals	Overall	
Afetr HM	87.6	11.1	0.7	0.7	-0.07	-0.04	65.839
After Part I	72.5	23.2	1.0	3.3	-0.51	-0.37	78.813

Figure 3 displays the PROSA profile calculated for the MC4R model generated by HM. The interaction energy per residue was calculated by the PROSA2003 program [36]. The knowledge based energies are below 0.5 suggesting high model quality. ProSA-Web analysis of developed MC4R structure shows the knowledge based energy graphs having negative values corresponds to stable parts of the structure shown in Fig. 4.
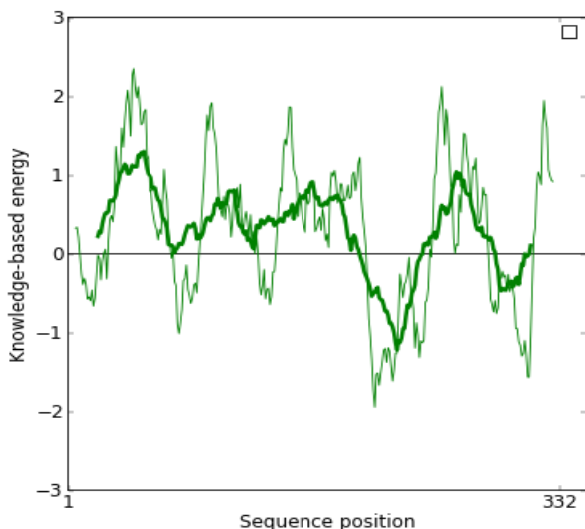
Fig. 4. Prosa energy plot for the MC4R.

Verify3D employs energetic and empirical techniques to generate averaged data points for each residue to evaluate the quality of 3D model of generated protein. This measure indicates the compatibility of the 3D model of protein with its sequence by means of a scoring function. The compatibility score above zero in the Verify3D graph is corresponding to acceptable side-chain environments (Figure 5). This suggests that the model has overall self-consistency in terms of sequence structure compatibility.
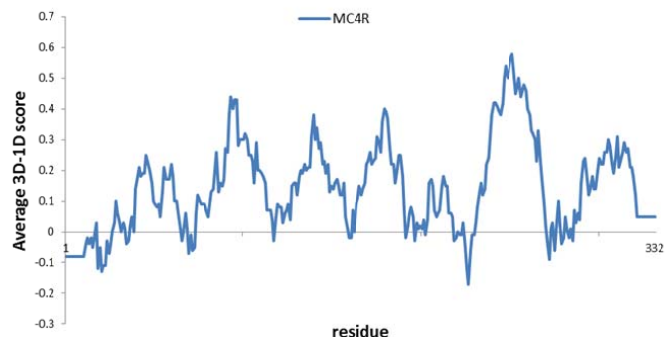
Fig. 5. The 3D profiles of MC4R model that was generated using Verify-3D server.

Overall compatibility score above zero indicates residues are reasonably folded It can be concluded that developed MC4R is a reasonable homology model, it has been obtained as a good representation of the actual system, and it can be exposed for examination of protein inhibitor interactions.

Molecular dynamics simulation

The developed model of MC4R was inserted in a pre-equilibrated lipid bilayer consisting of 414 molecules of POPC. Placing of the alpha helices of MC4R into the lipid bilayer core was carried out in such a way that alpha helices were perpendicular to the membrane plane and protein-overlapping lipids were removed. MDS procedure of the homology model of MC4R was carried out for two reasons: 1) to "refine" the developed MC4R model via simulation in an explicit bilayer plus solvent environment, by aiding identification of the less conformationally stable and hence (presumably) less well-modeled regions of the structure; and 2) to explore the nature of MC4R interactions with environment molecules.

Accordingly, the MC4R model was inserted into a POPC lipid bilayer that contained a hole of dimensions sufficient to accommodate receptor. Some minor adjustment of lipid molecules was needed to accommodate receptor by removing steric conflicts. The generated system was solvated, and counterions were added by replacing water molecules randomly, to provide an overall electroneutral system. To optimize the conformations of the lipids after receptor insertion, a 100-ps simulation was run, during which the protein atoms were restrained to their initial positions while the lipids and water were free to move, and temperature was raised to 310K (NVT ensemble). This was followed by an

equilibration run of 1 ns, during which the protein restraints were retained, but the pressure was reached to 1 bar along all three coordinate axes.

As a result of MDS procedure constraints between TM helices were reduced in sequential steps to avoid unwanted structural drifts during production phase of the MD. The POPC–MC4R system remained firm after the relaxation as very slight drift in energy, temperature, or lipids density was monitored during the MD (data not shown).

Fig. 5A shows the time history of RMSD for protein structure immersed in lipid bilayer relative to the starting structure (the output of the homology modeling process). As it is evident, C_α RMSD was not increased significantly after 7 ns of simulations. The initial rise in the C_α RMSD over the first 200 – 300 ps is common in such simulations and may be attributed to relaxation of the protein upon its transfer to a bilayer environment and/or inaccuracies in the potential function. The RMSD value implies that this protein structure has been affected by its environment dramatically. In Fig. 5B variation in total energy versus time in 10 ns of MD is illustrated, which shows small fluctuation in the last 5 ns of MD.

Furthermore, the average temperature in 10 ns of MD simulation at 310 K was equal to 310 ±1.16 K for the investigated system (Fig. 6B).
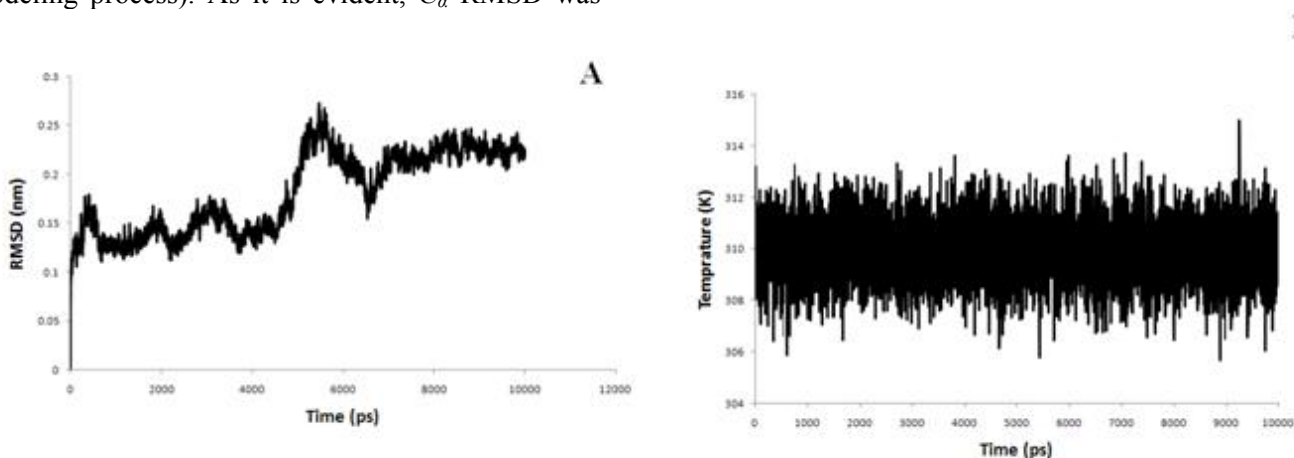
Fig. 6. (A) Time dependence of the RMSDs (Å) from homology model of MC4R for the C_α atoms in the 10 ns MD simulation (B) Time dependence of the temperature during the first phase of MD simulation.

Therefore, the extracted equilibrium structure at 310 K belonging to the MC4R was obtained under stable temperature conditions. These facts show that energy conservation was satisfied in MD simulation. The final conformation of the MC4R in POPC and water is illustrated in Fig. 7.
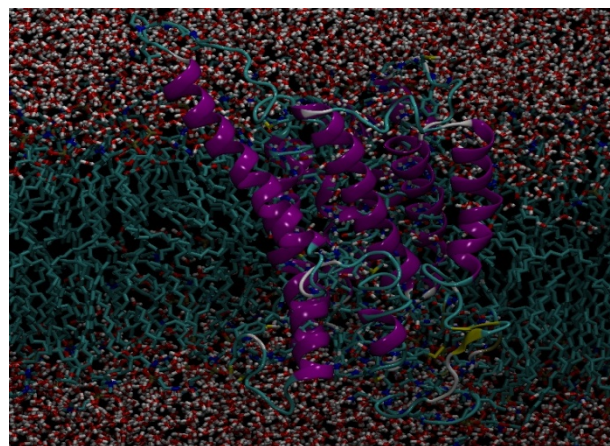

Fig. 7 MC4R in hydrated POPC lipid bilayer after 10 ns MD simulation, lipids in cyan and waters in red/white. The front half of the lipid bilayer and water molecules doesn't show for the sake of clarity.

After structural refinement of MC4R model by MD simulation, the geometric quality of the backbone conformation, namely all the tests performed in homology modeling step, was carried out again and the quality of the model was confirmed (Table 2 and Figure8).
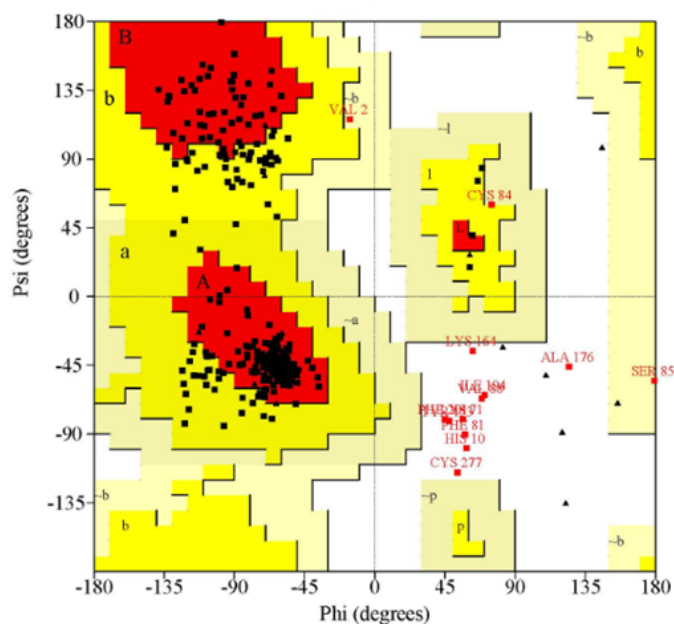

Fig. 8. Ramachandran plot of MC4R after MD simulation.

ERRAT is a so-called "overall quality factor" for nonbonded atomic interactions, and higher scores mean higher quality [37]. The normally accepted range is >50 for a high quality model [37]. In the current case, the ERRAT score is 78.813, well within the range of a high quality model and more than HM product. As can be seen in the Table 2 the ERRAT score for the MC4R after HM is 65.839. Thus, the backbone conformation and non-bonded interactions of the homology model and protein after MD part 1 are all within a normal range. Also, the final structure of MC4R indicates that more than 96.7% of residue φ-ψ angles are in the favored or additional allowed regions of Ramachandran plot (Table 2). With respect to Ramachandran plot, it is observed that only two residues (Ser85, Asn17) are in a disallowed region after HM phase. After MD, 9 residues (Lys164, Ala176, Ile194, Val88, Phe81, His10, Cys277, Tyr153, and Phe201) are located in the disallowed region (Figure 8).

In brief, the quality of the backbone geometry of the developed model, the residue interactions, the residue contacts, and the dynamic stability of the structure are within the limits established for the reasonably good model structures.

RMSF

The RMSF plot (figure 9) reveals a comparatively higher fluctuation of TM4 (about residue 168–186) than other TMs. In Overall, the movements of TMs, which surround by the lipid molecules, are lower than other parts of receptor..

From the RMSF plot, it can be seen that ICL3 (residues 218–244) and ECL3 (residues 268–283) exhibit increased fluctuations.

The radius of gyration, R_g, is defined here by

$$R_g^2 = \frac{1}{N} \sum_{i=1}^{N} (r_i - \bar{r})^2$$

where N, r_i, and $\bar{r}$ ($=\frac{1}{N}\sum_{i=1}^{N} r_i$) are the total number of the atoms in the protein investigated, the coordinate vector of atom i, and the center of geometry of the protein, respectively.
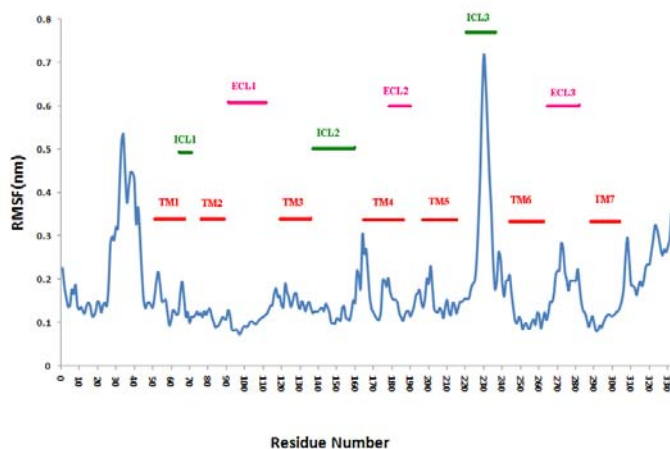
Fig. 9. RMSF plot of the C_α of amino acids; TM: transmembrane helix; ECL: extracellular loop; ICL: intracellular loop.

The fall in the radius of gyration values (Fig.10) reveals that the structure of MC4R becomes more compact in the presence of lipids.
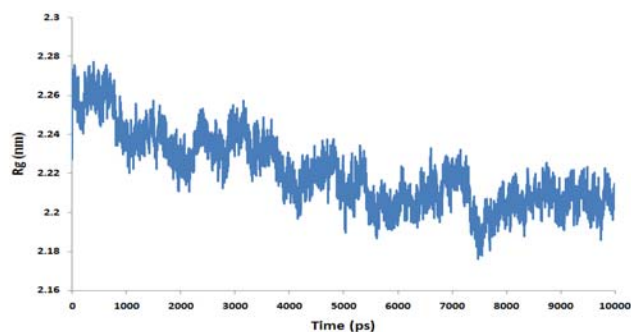

Fig. 10. Radius of gyration during 10 ns MDS.

Conclusion

Being a GPCR that is expressed in brain, MC4R poses a unique challenge as a system for study. The present study attempted to address several issues, starting from model building to MD simulation in the presence explicit lipid bilayer that are vital to the proper understanding of the actual mechanism of the functioning of GPCRs and that are also useful in the drug discovery process. The predicted structure of MC4R by using homology modeling was found to stand up to scrutiny, with about 87.6% of the residues falling within the most favored regions of the Ramachandran plot, while its quality was further refined and established through MDS procedure. The simulation results imply that the structure is stable in

an explicit POPC environment during the simulation, and that the helical conformations, a outstanding feature of GPCR super family, which remains parallel to the lipid bilayer were preserved.

The present work on MC4R sheds light on the structural aspects of an important GPCR, a famous protein family of vital medical importance. This computational homology modeling work in conjunction with MD simulations has successfully predicted the structure and provided insight into the structural characteristics of the MC4R and its dynamically changing conformations in the lipid bilayer.

This might help to design future experiments and further the understanding of this novel receptor.

Conflict of interests

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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