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Title:

Homology Modeling and Molecular Dynamics Simulations of the

Mu Opioid Receptor in a Membrane-Aqueous System

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Abstract

Three types of opioid receptors, mu, delta and kappa, belong to the rhodopsin subfamily in the G-protein-coupled receptor superfamily. With the recent characterization of the high resolution x-ray crystal structure of bovine rhodopsin, considerable attention has been focused molecular modeling of these transmembrane proteins. In the present study, a homology model of the mu-opioid receptor was constructed based on the X-ray crystal structure of bovine rhodopsin. A Phospholipid bilayer was built around the receptor and two water layers were placed on both lipid bilayer. Molecular surfaces of the simulations were carried out using CHARMM for the entire residues. system consisting of 316 amino acid 92 phospholipid molecules, 8327 water molecules chloride counter-ions (40,931 atoms altogether). The whole system was equilibrated for 250 ps followed by another 2 ns dynamic simulation. The opioid ligand naltrexone was docked into the optimized model and the critical amino acid for binding were identified. residues The mu opioid receptor homology model optimized in a complete membraneaqueous system should provide a good starting point for further characterization of the mu opioid receptor binding modes for opioid ligands. Furthermore, the method developed herein will be applicable to molecular model building to other opioid receptors as well as other GPCRs.

Keywords:

Molecular modeling, mu opioid receptor, membrane-aqueous matrix, membrane proteins, antagonist binding site

Introduction

Opioid receptors are members of the rhodopsin family of G-protein coupled receptors (GPCRs), which is characterized by a heptahelical domain spanning the plasma membrane. X-ray crystallography has confirmed the seven transmembrane (7TM) motif for rhodopsin. [1, 2, 3, 4] The 7-TM motif is believed to be an appropriate model for other GPCRs as well. [5, 6]

Rhodopsin X-ray crystal structure-based homology modeling has been carried out for GPCRs like, dopamine D3 receptor, muscarinic M1 receptor, Serotonin 5-HT₄ receptor, [7] metabotropic glutamate 5 receptor, [8] cholecystokinin CCK1 receptor, [9a] neurokinin-1 (NK1) receptor, [9b] human calcium sensing receptor (CaR), [10] and chemokine receptor

CXCR4. ^[11] Since the sequence identity in the TM domain of opioid receptors and rhodopsin is about 30%, it should be possible to build plausible opioid receptor models based on the rhodopsin X-ray crystal structure through homology modeling in which about 80% $C\alpha$ atoms are within 3.5 Å of their correct position. ^[12]

At least two methods have been developed to optimize homology modeling of GPCRs. One is to build a membraneaqueous system around the model and then conduct molecular dvnamics simulations for the entire protein-membrane-[11, 13] system. The aqueous second method information from site-directed mutagenesis studies relating to ligand binding affinities. Accordingly, specific amino acid residues can be constrained to counter moieties in the ligand at certain interacting distances and then molecular dynamics can be conducted to improve the protein structure. [9]

Here we report the construction of a mu opioid receptor homology model based on the X-ray crystal structure of rhodopsin and the optimization of the model in a complete membrane-aqueous system.

Results and Discussion

Homology model of the mu opioid receptor

Construction of the mu opioid receptor initial structure involved several steps: sequence alignment, assignment of Cartesian coordinates for amino acid residues in structurally conserved regions, assignment of coordinates for amino acid residues in structurally non-conserved regions, and refinement of coordinates for the backbone of the TM helices and correction with respect to hydrogen bonding orientations.

Homology modeling was the main tool to build the helices in the TM domain. For the EC region and IC region, homology modeling and protein database searching were combined to establish three-dimensional models. Since the N- and C- termini of the opioid receptors have longer chain lengths than that of rhodopsin, and no information on their conformation in the aqueous phase is directly available, these domains were partially truncated. Specifically, 35 amino acid residues at the N-terminus and 47 amino acid residues at the C-terminus were truncated and the new N-and C-termini were capped afterwards.

An analysis of the amino acid sequence identity of the three opioid receptors (mu, delta, and kappa) compared to rhodopsin, region by region, was carried out prior to homology modeling. Table I shows that all the TM domains of opioid receptors have a higher percentage of identity to

rhodopsin than the average value of the whole protein except TM3, which is believed to be an important contributor in the binding of opioid ligands. On average, all other TM helices among the three receptors have a sufficient percentage of identity for conducting homology modeling.

Since the opioid receptors have much longer N- and C-termini than those of rhodopsin, it is not surprising to find a very low identity in these regions for all three receptors. [13] Other regions of the opioid receptors having significantly low percentages sequence identity with rhodopsin domains are the extracellular loops (EL) 2 and 3, and the intracellular loop (IL) 3.

The importance of the extracellular (EC) regions of the receptors in the binding and recognition of opioid ligands makes it necessary to construct a model that includes those regions. On the other hand, the relatively low sequence identity between rhodopsin and opioid receptors in these regions makes it difficult to predict the exact conformation of EC regions of the target proteins by homology modeling. At the same time, loop modeling is typically applied to isolated loop regions of globular proteins, while loops of opioid receptors (as well as other GPCRs) have a number of unique tertiary interactions and

structural characteristics. Normally, geometric constraints are required to model the tertiary folding of those regions computationally. [14] Assuming the existence of the disulfide bond between EL1 and EL2 in the opioid receptor EC region, close proximity between these two loops can be expected. The intracellular (IC) region provides the recognition locus for G-protein, and its conformation is very important for the activation of the receptor. One possible way to verify the tertiary structure of the IC region would be to employ the x-ray crystal structure of heterotrimeric G-protein [15] as well as the proposed G-protein binding site structure of the IC region in rhodopsin. [16-18]

(Table I)

The initial and possibly the most essential step in homology modeling was sequence pairwise alignment between the mu opioid receptor and bovine rhodopsin using the InsightII/Homology module. Manual alignment of the amino acid residues was performed by comparing the chemical structures of the aligned residues in order to confirm the automatic alignment. The final alignment had 31.6% identity with the bovine rhodopsin sequence and the gap regions comprised about 8.5% of the total sequence.

The overlapping amino acid sequences between rhodopsin and the mu receptor were divided into different lengths separated by gaps. These regions are called "structurally conserved regions". The coordinates for the amino acid residues in these regions of rhodopsin were assigned to the corresponding residues in the mu opioid receptor. Up to 80% of amino acid residues in those regions were either identical, or structural related.

The amino acid residues of the mu opioid receptor outside of the structurally conserved regions constitute the "structurally non-conserved regions". The coordinates for residues in these regions were obtained by searching the protein database. The adopted conformation of these non-conserved regions all has close similarity to the backbone orientation of the corresponding regions in rhodopsin. If the segment was in the TM domain, the set of coordinates yielded a right-handed helix. Another critical step during this process involved the filtering the conformations that generated severe steric interactions with the structurally conserved regions.

In the process of assigning coordinates for structurally non-conserved regions, the helical backbone structures of the receptor may be kinked, bent or distorted. Such artifacts were removed by conformational

refinement using Discover/TemplateForce. Corrections for hydrogen bond orientation were carried out using Discover/GenericDis. After the proper TemplateForce and GenericDis constraint setup for a region, energy minimization and molecular dynamics were performed, while other part of the protein were constrained. Finally, the side-chain conformational energy of the entire protein was minimized with the backbone constrained until the energy deviation was less than 0.01 kcal/mol.

Compared with the models reported previously, [19] the mu opioid receptor homology model that we have constructed is more complete in that it contains the extracellular and intracellular loops to connect the TM helices along with a decent portion of the N- and C-terminal loops. Therefore it provides an improved starting configuration to study the interactions between different domains of the receptor. Also it is possible to study the roles of the EL loops of the receptor in binding affinity and ligand selectivity. [20, 21] The docking of selective ligands into the opioid receptor homology model may provide greater insight into ligand selectivity.

Construction of the receptor-lipid-solvent system

A complete membrane-aqueous system surrounding the constructed in order modeled receptor was to optimize the receptor structure. The primary reason for doing so was that the backbone of the TM domain in the receptor model was built based on that of rhodopsin. Furthermore, to avoid difficulties arising from the inherent conformational flexibility of the protein, homology modeling typically requires the use of geometric constraints. Consequently, the effects of the phospholipidbilayer-aqueous environment have been neglected in prior studies. In fact, it is important to understand the restrictions imposed on the membrane protein structure and the effects on tertiary structure prediction for membrane proteins by the lipid bilayers. Although the overall conformation of the TM domains with and without a membraneaqueous environment may be similar, it is possible that the refinement in such an environment may offer better opportunity to look into the tertiary arrangement of the receptor model. Such understanding may afford a more "dynamic" view of the receptor-membraneaqueous system.

Secondly, the conformation of the extracellular and intracellular loops of the receptor models may be better optimized in an aqueous environment along with the

transmembrane domain in a membrane matrix. In fact, as discussed in the literature, homology modeling may not be an ideal approach to predict the loop structures of GPCRs in the gas phase or even in continuum solvent. [22] Clearly, membrane-aqueous matrix is a more desirable choice than constrained energy optimization by fixing backbone atomic positions in the TM domain.

Finally, the conformation of hydrophobic side chains on the outer surface of the transmembrane domain can be more adequately optimized through molecular dynamics simulations in the membrane environment.

To our knowledge, there has been no literature report of a complete opioid receptor model in the membrane-aqueous system although a recent study reported a molecular dynamics simulation of an incomplete kappa opioid receptor in a DPPC bilayer system. [13] A more relevant example is the optimized homology model of the chemokine receptor CXCR4 in a lipid-bilayer-water environment. [11]

The program we adopted to build the membrane-aqueous-protein system was CHARMM (Chemistry at HARvard Macroolecular Mechanics) version c29b2. The method we followed was developed for the purpose of constructing an initial configuration of a protein-membrane complex that resembled a real membrane-aqueous system as much as

possible. We followed closely a procedure described by Woolf and Roux. $^{[23, 24]}$ The approach represents an extension of the work of Pastor and co-workers in the investigation of lipid bilayers. $^{[25, 26]}$

In the setup of the membrane system, pre-equilibrated conformers for each phospholipid 1, 2-dimiristoyl-SN-glycero-3-phosphorylcholine (DMPC) molecule were taken randomly from a set of 2000 that was previously generated from Monte Carlo simulations of an isolated DMPC molecule in the presence of a mean field. This molecule set was developed at 340K, above the gel-liquid crystal phase transition temperature. The structural characterization results calculated from molecular dynamics of the set agreed with results from solid-state NMR data of the liquid-crystalline state membrane. This was necessary because the available crystal structures of phospholipid molecules do not provide convenient configurations that can be used as a building block to assemble the protein membrane system. [27, 28]

In all, the complete membrane-aqueous system we constructed for mu opioid receptor homology model consisted of 316 amino acid residues, 92 DMPC lipid molecules, 11 chloride counterions, and 8327 water molecules. Altogether there were a total of 40,931 atoms in the system. The

dimensions of the central unit cell were approximately $80 \times 74 \times 60$ Å. After 200ps initial dynamics simulation to gradually release all the constraints in the system, another 2 ns MD simulation was conducted. A schematic representation of the entire system from a sliced side-view snapshot of the last configuration of the molecular dynamics simulations is shown in Figure 1.

(Figure 1)

In order to examine conformational variations of the receptor within the complex membrane-aqueous environment, the root-mean-square deviation (rmsd) with respect to the starting structure was calculated. Figure 2 shows the rmsd for $\text{C}\alpha$ atoms of the protein as a function of the simulation time (2 ns of the final molecular dynamics). The rmsd of $C\alpha$ atom rose to about 2 Å after 200 ps simulations and then leveled off after 500 ps. This indicates that after an initial increase in the magnitude of residue fluctuation, receptor protein reached equilibrium an characterized by the rmsd profile. In fact, the system was well equilibrated after about 700 ps of molecular dynamics simulation at 330 K based on the change of the total energy of the system. After the system was annealed to 310 K (from 330 K), the rmsd of the $C\alpha$ on the backbone of the protein was retained at about 2.6 Å relative to the starting structure.

(Figure 2)

Fluctuations of individual residues along the polypeptide chain were examined by the rmsd for the $C\alpha$ atom of each residue separately (Figure 3). As expected, the TM helical domain and the EL and IL loop regions had markedly different dynamic behavior, in that the average rmsd values in TM was about 0.5 Å, whereas the loop regions had rmsd fluctuations in the range of 1-2 Å. The largest fluctuation was observed for the N-terminus. Residues in the IL1 and EL1 segments also showed a somewhat higher degree of variation. Overall, the rmsd fluctuations suggest that the 7TM helical structure of the receptor protein is reasonably stable in the membrane-aqueous environment.

(Figure 3)

In summary, the mu opioid receptor homology model has been optimized in a complete membrane-aqueous matrix. After 2 ns dynamics simulation, the conformation of TM domain and loop

domains of the receptor model reached a newly equilibrated status in the matrix. Figure 4 shows the conformation change of the homology model before and after the dynamics simulation in the membrane-aqueous system.

(Figure 4)

First, the arrangement of α -helices in TM domain became more compact, which reflected the impact exerted by the lipid bilayer. Significantly, the $C\alpha$ atoms in the upper portion of TM3, TM5 and TM6, three critical helices that comprise the ligand binding pocket, shifted inward from 0.2 to 3.8 Å. This shift yielded a more compact binding pocket for opioid ligands and may provide more information about the interactions of the ligand with amino acid residues in the binding site. Secondly, the EL and IL domains of the receptor have been dramatically rearranged in the aqueous layers, which could not be achieved reasonably in the rearrangement should help This us to vacuo. understand the effect of the loop domains to the structure of the whole receptor. Given these differences, the dynamics simulation of the receptor model in a membraneaqueous system has produced a plausible starting structure for further optimization.

Molecular dynamics simulation of the mu opioid receptor docked with antagonist NTX

After an initial round of energy minimization and molecular dynamics simulation of the receptor homology model in the membrane-aqueous environment, the universal opioid antagonist, naltrexone (Figure 5), was docked into the cavity that has been identified as the binding pocket of the mu opioid receptor. [29] Energy minimization and molecular dynamics were then carried out for the ligand-receptor system to relax and optimize binding interactions between the ligand and amino acid residues in the binding cavity. Since the homology model was built based on the inactive state conformation of rhodopsin, using an antagonist in the docking process is more appropriate.

(Figure 5)

The ligand-receptor complex structure obtained after 11ps of molecular dynamics is depicted in Figure 5. In this structure, the distance between the protonated nitrogen atom of NTX and the carboxyl group of Asp147 was initially anchored at 4.5Å and retained at this value by a weak harmonic restraint during the molecular dynamics simulation

to represent the putative salt bridge that has been inferred from experimental studies. $^{[30]}$ In the final conformation of the complex after molecular dynamics simulation, the distance (4.3Å) was compatible with the initial setting.

Figure 6 shows that the binding pocket of mu opioid receptor to NTX has at least three binding domains. First, a polar and aromatic domain, which is composed of Asp147, Phe289, Trp293, Cys321 (not shown for clarity), and Tyr326, is in the vicinity of the protonated amino moiety and the 14-hydroxyl group of NTX. Besides the putative salt bridge between Asp147 and the protonated amino moiety of NTX, a cluster of aromatic side chains (Phe289, Trp293 and Tyr326) bind with the amino cation through cation- π may interactions. [31] At the same time it has been suggested by site-directed mutagenesis that the polarity of Tyr326 may be very critical for the recognition of NTX in mu opioid receptor binding pocket since the substitution of tyrosine residue to phenylalanine decreased the binding affinity of naltrexone. [32]

The second binding domain may be characterized as a number of hydrophobic interactions in the binding pocket, involving the phenolic moiety of the ligand and aromatic residues of Tyr148 from TM3, Tyr210 and Phe221 from ELII.

It has been reported that Tyr148 may have $\pi-\pi$ interaction with the phenolic moiety of morphinane opiates. [33]

There is another hydrophobic domain which involves Trp318, Leu219, Ile322, Ile296, and Ile144 (not shown for clarity). The domain may have hydrophobic interaction with the aliphatic piperidinyl and cyclohexanonyl skeleton in NTX. As verified by site-directed mutagenesis, Trp318 may play a critical role in the binding of selective mu opioid receptor ligands, such as morphine and NTX while they had lower affinity to mutant mu opioid receptor W318L and W318K. [34]

(Figure 6)

Conclusions

We have constructed a homology model of the mu opioid receptor based on the X-ray crystal structure of bovine rhodopsin and further refined the model by molecular dynamics simulations in a membrane-aqueous environment. [35] An opioid receptor antagonist, naltrexone, was then docked into the receptor model and specific ligand-receptor interactions were suggested after molecular dynamics simulations. The homology model may provide a more robust model of the receptor structure for analysis of specific

binding interactions with the opioid ligands. Optimization of the mu opioid receptor model in the membrane-aqueous system has provided a more compact binding pocket which accommodates naltrexone binding cavity. The present mu opioid receptor model may be the first step toward the construction of an interaction complex involving the receptor, an agonist and G-protein in a membrane matrix to gain insights into receptor activation and structural transformation.

Experimental Section

The sequence of mu opioid receptor was obtained from NCBI Database (ID code: AAE87936). Homology modeling was carried out using the Homology and Discover modules in the InsightII package. All computations were performed on SGI Origin 2000 or Octane2 workstations (Silicon Graphics, Inc., Mountain View, CA), and an IBM SP system with 8 processors at the Minnesota Supercomputing Institute.

The general strategy for creating a reasonable starting configuration for the protein-phospholipid system consisted of randomly selecting lipid molecules from a preequilibrated and pre-hydrated set, placing them around the protein, and finally reducing the number of core-core overleaps between heavy atoms through systematic rotations

about the Z-axis, which is along the norm of the membrane plane, and translations in the XY plane of the lipids and protein. To provide the initial positions for each lipid molecule in the XY plane, the whole lipid molecule was first represented by a single effective van der Waals sphere, corresponding to the average phospholipid cross-section area. The packing of the effective lipid particles around the membrane protein was optimized from a molecular dynamics simulation in which the large effective particles were harmonically restrained along the Z direction, but were allowed to freely move in the XY plane. Periodic boundary conditions were used in all calculations. The resulting Cartesian positions of the large spheres were utilized for the initial placement of the phosphate head group of each phospholipid.

The pre-equilibrated conformers for each phospholipid 1,2-dimiristoyl-SN-glycero-3-phosphorylcholine (DMPC) molecule were taken randomly from a library of 2000 that was previously generated from Monte Carlo simulations of an isolated DMPC molecule in the presence of a mean field (this library is distributed along with the program CHARMM). [25, 26, 36] The conformers generated by the mean-field Monte-Carlo simulations, in so far as they agreed with the available experimental data, were representative of the

phospholipid molecules found in a bilayer membrane in thermal equilibrium with its normal axis in the Z direction. In order to provide the primary hydration for the polar headgroup, approximately twenty water molecules around both the phosphate and the choline group were also included in the library; these water molecules were obtained from snapshot configurations during molecular dynamics simulations of o-phosphorylcholine (o-PC) in bulk solution. [26]

Because the initial configuration was first assembled with totally uncorrelated phospholipid conformers, there were a large number of unrealistic overlaps. To improve initial configuration, a global search, using the systematic rigid body rotation and translation of the lipids (along with the primary companion waters), was performed to reduce the number of bad contacts. Systematic rotations were performed with rigid body rotations around the Z-axis in 10-degree increments for each individual Systematic translations in the XY plane were lipid. performed with 0.25-Å steps around a square of side 2 Å centered at the initial position of each lipid. A "bad contact" was defined as the presence of a distance of less than 2.6 Å between two non-hydrogen atoms of the system. After the global search based on systematic rotation and

translation, the remaining contacts were removed by energy minization. For each cycle, the protein was fixed and the van der Waals radii of the lipids were gradually increased from zero to the final force-field values.

To obtain a solvated microscopic system, the remaining bulk solvent was built using a previously equilibrated box of water molecules. The water box was translated along the Z-axis and its position was adjusted to match the interface with the lipid bilayer. Water molecules that are within 2.5 Å from any protein or lipid non-hydrogen atoms and those that penetrate into the hydrocarbon interior of the bilayer by 12 Å were deleted. After the operation, energy minimization was performed for 200 steps to remove close contacts.

The equilibration of the protein-membrane-solvent system consisted of three different stages. In the first stages, we carried out molecular dynamics simulations using the Langevin equations of motion for 200 ps at 330 K after the system was gradually heated to the final temperature. Initially, the peptide backbone atoms and the centers of mass of the phospholipid head groups were restrainted by harmonic forces. The harmonic restraints were gradually decreased, so that by the end of 200 psec, the full system was completely free. The Langevin dynamics was followed

additional 50 ps without any constraints. Newtonian molecular dynamics simulations were performed to further equilibrate the system for 1 ns. The first 960 ps simulation was carried out at 330K for rapid equilibration relaxation of the system. We then lowered temperature from 330 K to 310 K in 20 ps, and this was followed by an equilibration of 20 ps more at 310K (making a total of 1 ns of equilibration in stage 2). stage consisted of 1ns molecular dynamic simulation at 310 K, from which the coordinates for 1000 configurations from the trajectory were saved and used for analysis. calculations were carried out using the isothermal-isobaric ensemble at 1 atm and 310 K (or 330 K in the beginning).

The coordinates for the ligand molecule NTX were created using InsightII. The conformation of the compound was subsequently optimized, relaxed, and equilibrated by molecular dynamic simulations in aqueous solution at 300K. The ligand was modeled in its nitrogen-protonated form. We used the final structure in water from the 2 ps molecular dynamics calculations, which is as arbitrary as any other configurations explored during the simulation, as the initial configuration for docking into the proposed binding site of the mu opioid receptor. Experimental studies [37] suggest that the protonated nitrogen moiety interacts with

the carboxyl group of Asp 147 to form a putative salt bridge. The phenol ring was placed in the binding pocket to provide the most effective π - π stacking and cation- π interactions [38] with appropriate aromatic amino acids. The rest of the molecule was oriented toward the extracellular surface. Then the position of the ligand was adjusted to avoid overlap with the receptor atoms.

Molecular dynamics simulations of the ligand and receptor complex were performed in gas phase first with the backbone of the receptor fixed, but all the side chain atoms were left unconstrained. The optimized conformation was then used as the initial configuration for the dynamics simulations including solvent, which was modeled by a layer of 20 Å of water molecules above the lipid bilayer. Steepest descent energy minimizations were performed for 2000 iterations, after which 10000 steps dynamics was conducted with 1000 steps equilibration for the initial dynamics. The total simulation time was 11 ps.

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Table I Comparison of the Amino Acid Sequence Homology of Opioid receptors with Bovine Rhodopsin by Domain

Percent Sequence Identity Compared with

Bovine Rhodopsin *

Domain	Mu	Delta	Kappa
TM1	32	32	20
TM2	44	36	44
TM3	13	17	13
TM4	28	24	28
TM5	44	36	40
TM6	42	40	42
TM7	32	32	30
EL1	36	27	36
EL2	14	14	13
EL3	15	17	18
IL1	36	45	27
IL2	37	37	32
IL3	18	15	11
N-terminus	6	14	10
C-terminus	15	16	13
Entire	26	23	25
protein			

^{*} Comparison and calculation of the sequence identity percentage was conducted after pair alignment of the whole protein with rhodopsin. Numbers in the columns refer to the percent of amino acid residues within a particular domain, or for the entire protein, that are identical after a comparison of the specific opioid receptor with rhodopsin.

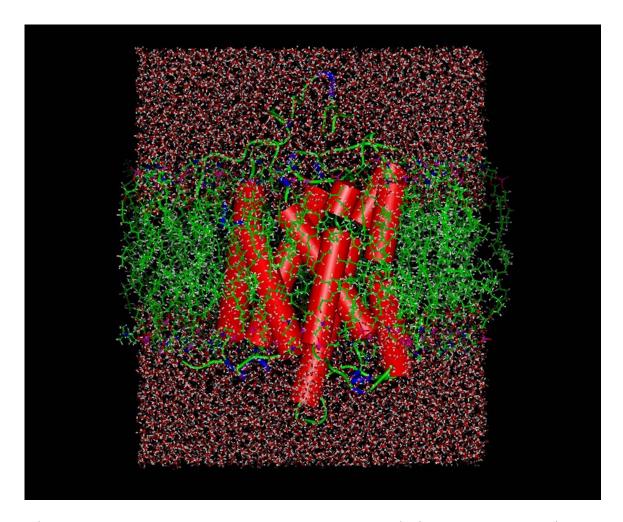


Figure 1 Homology model of Mu opioid receptor in a membrane-aqueous system.

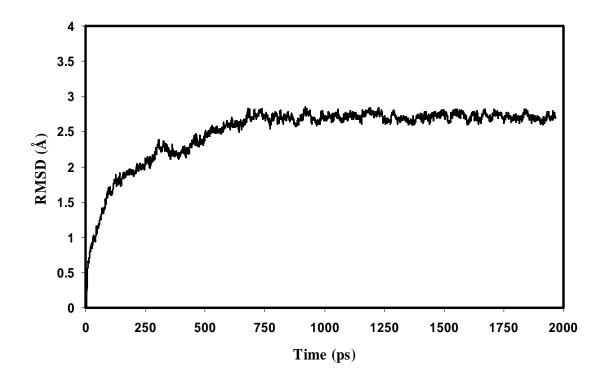


Figure 2. RMSD versus simulation time for the $\text{C}\alpha$ atoms of the mu opioid receptor relative to the starting structure.

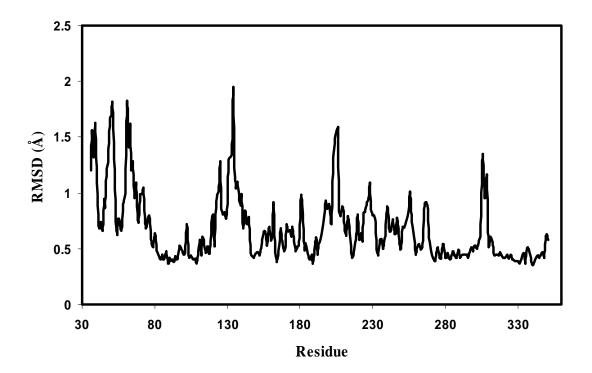


Figure 3. Residue-by-residue $\text{C}\alpha$ rms fluctuations about their average coordinates of the receptor.

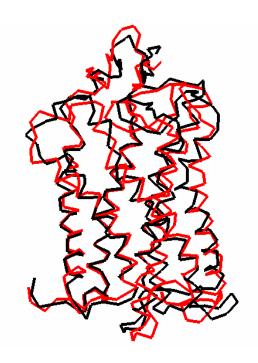


Figure 4. Superimposition of the mu opioid receptor homology models before (red) and after (black) the dynamic simulation in the membrane-aqueous system.

Naltrexone (NTX)

Figure 5. Chemical structure of opioid receptor antagonist naltrexone.

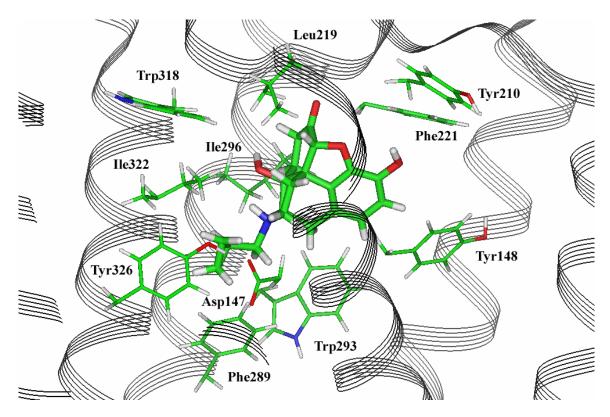


Figure 6. Characterization of mu opioid receptor antagonist binding site by docking of naltrexone into the binding cavity.