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Conformational Heterogeneity of Intracellular Loop 3 of the μ -opioid G-protein Coupled Receptor

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Abstract

G-protein coupled receptors (GPCRs), including the μ -opioid receptor, interact with G-proteins and other proteins via their intracellular face as required for signal transduction. However, characterization of the structure of the intracellular face of GPCRs is complicated by the experimental methods used for structural characterization. In the present study we undertook a series of long-time molecular dynamics (MD) simulations, ranging from 1 to 5 μ s, on the μ -opioid receptor in both the dimeric and monomeric states. Results show intracellular loop 2 (ICL2) to sample an equilibrium between coiled and helical states. Intracellular loop 3 (ICL3) samples a wider range of conformations. Previously unobserved β -sheet structures were primarily sampled in the simulations initiated from the inactive dimer conformation. In contrast, helical structures were sampled in simulations initiated from the active, monomer conformation. Notably, in the dimeric form of the receptor, both intramolecular and intermolecular β -sheet structures were sampled, with the latter occurring between the two monomers. These results indicate that the sampling of β -sheet structures can maintain the ICL3 in an inactive conformation that contributes to stabilization of the dimeric form of the receptor via interchain β -sheet structures.

Graphical Abstract

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Supporting Information. RMSDs of the receptors and the ligands along MD simulations, 2D PMFs characterizing the conformational change of the OR, probability distributions of ligand-Asp147 distance, and the Direct-ID analysis result.

Introduction

Opioids are the oldest known analgesics, and are still being prescribed to alleviate severe pain in spite of their adverse effects.¹ The receptors of opioids belong to the family A G-protein coupled receptors (GPCRs). GPCRs are membrane proteins containing seven trans-membrane (TM) helices that couple to heterotrimeric G as well as other proteins on the intracellular face.² Upon ligand binding, conformational transitions in GPCRs are transmitted to a range of G proteins that cause activation.³ There are three traditionally recognized types of opioid receptors (ORs), μ , κ and δ , as well as the nociceptin/orphanin FQ receptor, and a large variety of ligands are known to target them.⁴ Understanding the structural features of the ORs, as well as GPCRs in general, that activate specific G-proteins is important for molecular structure activity relationships (SAR) and for ligand design efforts, such as UMB 425 developed in our laboratory,^{5, 6, 7} a dual-function μ -OR agonist and δ -OR antagonist opioid with reduced tolerance liabilities.⁸

Structural understanding of the SAR of GPCRs has been driven by novel methods to obtain crystals of these proteins. This has lead to a general understanding of the conformational changes the proteins undergo upon activation, though details of the activation mechanism of specific GPCRs and subtle details of those mechanisms are still lacking. In 2012 the crystal structure of the μ -OR was solved with the covalently bound antagonist β -funaltrexamine (β -FNA) with the protein present as a two-fold symmetrical dimer.⁹ The structures of κ -OR¹⁰ and δ -OR^{11, 12, 13} have also shown them to be dimers in the presence of antagonists and in their inactive conformations. More recently the crystal structure of activated μ -OR was reported.¹⁴ The structure includes the agonist BU72 and a G protein mimetic nanobody bound to the intracellular face and the receptor in a monomeric form. While these studies have yielded insights into the relationship of the conformation of the TM helices to activation in the ORs our understanding of the conformational properties of the intracellular loops, which may potentially be impacted by the constructs used to facilitate crystallization, are poorly understood. The difference of the μ -OR structures being in dimeric or monomeric forms is also interesting, as emerging experimental evidence show that a number of GPCRs

form homodimers, heterodimers, or high-order oligomers *in vivo*.^{15, 16} Dimer formation is essential for the function of family C GPCRs such as glutamate receptors,¹⁷ while family A GPCRs including opioid receptors can activate G proteins both as monomers and as oligomers.^{18, 19} The functional and pharmacological implication of GPCR oligomerization is poorly understood and an ongoing area of research.¹⁶

In 2013 Shim, Coop and MacKerell presented the first atomistic simulations of an OR, focusing on the μ -OR dimer in which a variety of ligands were modeled into both binding sites.²⁰ Simulation results confirmed our earlier ligand-based SAR studies employing the conformationally sample pharmacophore method.^{5, 6, 7} In addition, the distance between the basic N of the ligands and the sidechain of Asp147 along with its χ_1 dihedral were shown to be related to agonism vs. antagonism and results from the simulations explained the long-known but unexplained impact of the size effect of the substituent on the basic N on the activity, i.e., small substituents are associated with agonism, medium size with antagonists, but longer substituents are again agonists.²¹ Yuan et al subsequently carried out several 1.4 μ s MD simulations of μ -OR bound with the agonist morphine and the antagonists naltrexone and naloxone, and analyzed the distribution of water and sodium ions.²² They found that antagonists block the exchange of water molecules between the extracellular space and the sodium allosteric binding site. A further study by Yuan et al involving 3 μ s simulations of μ -OR and κ -OR bound with the agonist morphine and antagonist levallorphan confirmed the disruption of water pathway by specific interactions between the antagonist and the ORs.²³ Shang and coworkers performed up to 1 μ s MD simulations of several μ -OR, κ -OR and δ -OR systems, with the analysis also focused on the binding and allostery of sodium ions.²⁴ Fossepre et al reported a 0.5 μ s MD trajectory of apo μ -OR, and analyzed the flexibility, bendability and coupling between its TM helices.²⁵ All these simulations started from the μ -OR inactive structure⁹ and described the opioid receptors as a monomer in the membrane environment.

Here, we extend simulation studies of the μ -OR by performing simulations of monomeric and dimeric states on the time scale of 1 to 5 μ s in the presence of both agonists and antagonists as well as on the T297K constitutively active mutant and the apo form of the protein. While results indicate that the conformational changes involving the TM helices do not occur on the 1 to 5 μ s time scale, the simulations did extensively sample the conformational properties of the intercellular loops, indicating the presence of previously unobserved β -sheet conformation in intracellular loop 3 (ICL3). While helical conformations were observed in the monomeric, active form, β -sheet structures were observed in the dimeric, inactive states of the protein with, notably, interchain β -sheets between the monomers observed in the dimer simulations. Such previously unobserved local secondary structural heterogeneity offers a prediction of a mechanism by which ICL3 can contribute to dimerization of the μ -OR thereby stabilizing it in the inactive state.

Methods

1. System Preparation and MD Simulations

Six μ -OR dimer simulations were carried out on Anton, including three agonist bound systems (morphine, DAMGO and etorphine), two antagonist bound ones (β -FNA and

naloxone), and the constitutively active T279 mutant. Starting structures for simulations were prepared based on the last frames from the previously reported 140 ns MD simulations.²⁰ Briefly, the initial structures was built using the crystal structure of μ -OR (pdb id: 4DKL)⁹, with missing residues in the third intracellular (ICL3) loop restored and explicitly represented. One disulfide bond is present between Cys140 and Cys217 for each monomer. The lipid bilayer was composed of 216 POPC and 24 cholesterol molecules, resulting in a 9:1 ratio, and all systems were solvated with 0.15 M NaCl. The starting coordinates for ligands were determined by the RMS alignment using ligand pharmacophoric fragments with the known binding structure of β -funaltrexamine (β -FNA) which is co-crystallized with the μ -OR and solved in the crystal structure. The placement of morphine, etorphine, and naloxone was based on aligning their common 4,5-epoxymorphinan scaffold with β -FNA, while the superposition of DAMGO was based on the overlaying it with etorphine by matching the Tyr residue of DAMGO with the phenol ring of etorphine, the N-terminal amino group with the basic nitrogen, and the Phe residue with the C19 substituent. The bound orientations from the final snapshot of the previously described 140 ns MD simulations²⁰ were used to initiate the present simulations. As Anton only supports orthorhombic periodic boxes while the previous simulations were performed with hexagonal symmetry, periodic image atoms of these hexagonal systems were built in the x,y directions that are parallel to the membrane plane and then trimmed into a 94 Å * 94 Å square. This leads to tetragonal systems with a size of about 94 Å * 94 Å * 116 Å, and increases the total number of atoms from about 90,000 to about 105,000.

The receptor and lipid are modeled with the CHARMM36 (C36) protein²⁶ and CHARMM36 lipid force field.²⁷ We note that for cholesterol the original C36 parameter set²⁸ was used instead of the more recent C36c parameters,²⁹ as the NBFIX terms introduced in C36c significantly slow down Anton simulations. Opiate molecules were modeled using CHARMM General Force Field (CGenFF),³⁰ and an in-house python script was used to convert CGenFF toppar stream files into the vipar format for Anton. The CHARMM-modified TIP3P water model is used.³¹

Simulations were initially carried out using NAMD³² in the isothermal–isobaric (NPT) ensemble at 303.15 K and 1 ATM for 50 ns as equilibrium. Periodic boundary conditions were applied and Lennard-Jones (LJ) interactions were truncated at 12 Å with a force switch smoothing function from 10 Å to 12 Å. The non-bonded interaction lists were generated with a distance cutoff of 16 Å and updated heuristically. Electrostatic interactions were calculated using the particle mesh Ewald (PME) method³³ with a real space cutoff of 12 Å on an approximately 1 Å grid with 6th order spline. Covalent bonds to hydrogen atoms were constrained by SHAKE.³⁴ The coordinates, velocities, and periodic boundary information after NAMD equilibrium runs were then converted into Anton format and MD simulations continued for 5.04 μ s in the canonical ensemble on the Anton machine. Coordinates were saved every 240 ps.

In addition, 1 μ s MD simulations for several μ -OR monomer systems were performed using both inactive and active conformations. From simulations starting from selected dimer simulation snapshots, the coordinates of the protein and ligand were taken and all the water molecules and sodium ions located inside the receptor were preserved. Then the lipid

bilayers as well as water and ion solvation were rebuilt using the CHARMM-GUI.^{35, 36} Simulation systems starting from the active crystal structure (pdb id: 5C1M)¹⁴ included a morphine bound and an *apo* one. Each of these systems were first energy minimized using 1500 steps of steepest descent (SD) and another 1500 steps with the adopted basis Newton-Raphson (ABNR) algorithm. During minimization, the non-hydrogen atoms of the protein backbone and side chains were harmonically restrained using force constants of 10 and 5 kcal/mol/Å², respectively. Influx of water molecules into the hydrophobic core of the transmembrane (TM) region was prevented using a harmonic restraining potential of 2.5 kcal/mol/Å² along the x-y plane at positions of 11 Å along the z-axis from the center of the receptor. The same restraining force was also used to keep the heads and tails of the lipids in place, and configurations of the lipids were maintained with harmonic dihedral restraints with a force constant of 250 kcal/mol/rad². The same restraints were used during the 375 ps of equilibration with a 1-fs time step, and the restraint forces were gradually reduced as described in our previous work.³⁷ The first 50 ps of equilibration was performed using Langevin dynamics with a friction coefficient of 3 ps⁻¹ on all non-hydrogen atoms. The remaining 325 ps of the equilibration used constant pressure-temperature (NPT) dynamics using the Langevin Piston integrator. Covalent bonds involving hydrogens were fixed at their equilibrium lengths by the SHAKE algorithm. Lennard-Jones (LJ) interactions were switched to zero from 10 to 12 Å and the non-bonded pair list was generated out to 14 Å and updated heuristically. Electrostatic interactions were calculated by the PME method with a real space cutoff of 12 Å. These minimization and equilibration simulations were performed using CHARMM. Following equilibration, production runs were performed using GROMACS³⁸ with the leapfrog integrator (GROMACS integrator “md”) and a time step of 2 fs. The Nose-Hoover method was used to maintain the temperature at 300 K with the protein and the remainder of the system separately coupled to heat baths. Pressure was maintained at 1 bar using the Parrinello-Rahman barostat. The time constant used for temperature and pressure coupling were both set to 1 ps. The LINCS algorithm was used to constrain all covalent bonds involving hydrogen atoms and water geometries were kept rigid with SETTLE. LJ interactions were switched off smoothly in the range of 10–12 Å, and the PME method was used to treat long-range electrostatics with cubic B-spline interpolation and a maximum grid spacing of 1.2 Å. Long-range dispersion correction was applied to the energy and pressure terms. No restraints were applied during the production simulations.

2. Trajectory Analysis

Identifying structural transitions in long timescale biomolecular simulations is a challenging task.³⁹ Recently we developed an automated method called DIRECT-ID (Dimensionality Reduction through Covariance matrix Transformation for Identification of Differences in dynamics) that is able to quantify the differences in macromolecular conformational dynamics and extract structural features with significant differences in conformational motion across two simulations.³⁷ The method is based on comparing the norm of the differences in the covariance matrices calculated from multiple independent MD trajectories. Briefly, one computes a covariance matrix (C) for each of the M given trajectories. Difference matrices ($D_{ij}=C_i-C_j$) are calculated for each pair of trajectories, i & j . Norm of the difference matrix is calculated as the maximum absolute column sum of D_{ij} . Thus, differences between two trajectories can be effectively represented as a one-dimensional

quantity, instead of the $3N \times 3N$ matrix (N being the number of atoms in the system). Each of these norm quantities can then be binned into a $M \times M$ matrix to comprehensively represent the differences in conformational motion across all the M trajectories. For trajectories with significant differences, characterized by a high value of norm, the difference matrices are parsed to identify atom pairs with the largest changes in the correlated motions between two trajectories. The final output is a list of residues with the most prominent changes in their dynamics between the two trajectories. The utility of the method was shown in a study of the β_2 -adrenergic receptor (β_2 AR), a prototype class I GPCR.

To characterize the conformational change related to activation, two-dimensional (2D) potentials of mean force (PMF) were calculated. These involved the distances between the TM2 and TM6 helices (H2–H6), and between the TM3 and TM7 helices (H3–H7) using probabilities obtained with a bin size of 0.1 Å. Residues used for the calculations were Ile105-Asp114 (H2), Ser154-Val163(H3), Arg276-Val285 (H6), and Cys330-Leu339 (H7).

Results and Discussion

Motivated by the observation in our previous simulation study of the μ OR in the presence of a of selected agonists, antagonists, a partial agonist and the T279K mutant we undertook long-time MD simulations of a number of those complexes, which were based on the original dimer inactive form of the μ OR, taking advantage of the Anton supercomputer. Analysis of those simulations showed them to be stable based on root-mean-square differences (RMSD) of the individual monomers with respect to each other (Fig. S1), the change in the solvent accessible surface of the dimer interface (Fig. S2) and the 2D potentials of mean force of the distances between the TM2 and TM6 helices (H2–H6), and between the TM3 and TM7 helices (H3–H7) (Fig. S3 and S4). The conformations of ligands were also stable and the ligands remained buried in the binding pocket throughout the simulations (Fig. S5 and S6). The stability of the dimer interface is consistent with previous coarse grain simulation studies in which the TM5:TM6 interface in the crystallographic dimer was shown to be present.^{40, 41} The lack of conformational changes in the agonist-related dimer simulations is consistent with a comparison of the inactive dimer and active monomer crystal structures¹⁴ indicating that steric clash of TM5:TM6 dimer interface will hinder outward movement of TM6 required for receptor activation.

The lack of significant conformational changes in the dimer simulations as well as the more recent crystal structure of the active form of the μ OR being a monomer motivated simulations of selected complexes in the monomeric form. Three structures from the dimeric simulations were selected in which the conformation diverged the most from the inactive form towards the active form (Fig. S4). Monomers from these structures were extracted, the full bilayer solvated systems rebuilt and subjected to 1 μ s simulations. As with the full dimer simulations, significant conformational changes were not observed based on 2D PMF analysis (Fig. S4B). In addition, two monomeric simulations initiated from the active monomer crystal structure maintained the active conformation after 1 μ s including a simulation of the apo form of the protein. Thus, extending the dimeric simulations to the 5 μ s time range and carrying out additional 1 μ s monomeric simulations from inactive conformations did not yield insights into larger conformational changes in the receptor

leading to the active conformation, consistent with the previously reported simulation studies of the μ OR.^{22, 23} However, the results are consistent with the previously observed trend for agonists to have much higher probabilities to sample shorter D147 sidechain to basic N distances than antagonists. As shown in Fig. S7, agonists have higher probabilities of direct interaction between nitrogen and Asp147, while antagonists have lower probabilities (β -FNA) or no such interaction at all (naloxone), confirming that the interaction with Asp147 is an important pharmacophore feature to be exploited in designing better opioids. The probability distributions differ between two monomers, indicating that the asymmetry in the changes occurring in the receptor on the 5 μ s time scale (Fig. S3) propagates into asymmetry in the ligand-protein interactions.

While overall global changes in the OR were not sampled on the time scales of the present simulations DIRECT-ID analysis³⁷ indicated significant structural differences in intracellular loops 2 and 3 (ICL2, ICL3), with noticeable differences between the monomer and dimer simulations in ICL3 (Fig. S8). Accordingly, detailed structural analysis of the loops was undertaken with emphasis on the sampling of secondary structures using the dictionary of secondary structure of proteins (DSSP)⁴². The resulting secondary structure versus time data are shown in Fig. 1 for the dimer simulations and Fig. 2 for the monomer simulations.

The ICL2 loop, which has a helical conformation in the crystal structure, expectedly samples a significant amount of α -helices on the microsecond timescale. In the crystal structure the helix is comprised of residues P172 to R179. In the simulations a diverse range of behavior is observed with the most stable helical segments including A175 to F178 (Fig. 3A), with the residues at the end of TM3 (P172-V173-K174) (Fig. 3B) intermittently sampling helical states. In the dimers, the helical content in the majority of cases is lost within the first 1 μ s though in some cases, including the B chains for the morphine-, etorphine- and β -FNA-bound states, it is maintained throughout the full 5 μ s. However, in the monomer simulations the full helix is sampled throughout the 1 μ s simulations. These results are consistent with a number of x-ray structures of GPCRs where both coiled and helical conformations have been observed, and with the ICL2 helix formation and disruption on the μ s timescale that was previously observed in simulations of the β 2 adrenergic receptor.^{43, 44} Thus, ICL2 rapidly interconverts between coiled and helical states on the sub microsecond time scale, with the present results indicating the monomer form to favor the helical state.

In contrast, the ICL3 loop adopts both β -sheet and helical secondary structures in the present simulations. In the dimer simulations β -sheet conformations are primarily sampled with a very small amount of helix sampled in the majority of simulations. However, in the monomer simulations initiated from the active, monomeric crystal structure a helical conformation of ICL3 is sampled (Fig. 2D and E). These helices are located at the N terminus of TM6 (K269 to N274) and can be considered as the continuation of TM6. Helical sampling also occurred in the morphine-bound monomer simulation initiated from the dimer simulation (G267 to E270, Fig. 2C); the time frame from the dimer simulation used to initiate that simulation has ICL3 in the helical state, though when the dimer simulation was continued the conformation switched back to β -sheet (Fig. 1A). In the etorphine-bound monomer simulations β -sheet was predominant (Fig. 2B and C), with both of those

simulations initiated from time frames from the dimer simulation in which the β -sheet was present. Comparison of morphine-bound and etorphine-bound simulations showed that the helices in ICL3 were stabilized by a salt bridge interaction between Arg273 and Asp340 at the junction of TM7 and helix 8 (H8). Although multiple secondary structure formation and breaking events were observed in the simulations of different systems, we note that the simulation timescale is still not long enough to obtain quantitative information on the equilibrium of the conformational dynamics.

Interestingly analysis of ICL3 in the dimer simulations showed that both interchain and intrachain β -sheets were occurring. Shown in Figure 4 and 5 are time series of the sampling of the intrachain and interchain β -sheet structures, respectively. Examples of the intrachain and interchain β -sheets are shown in Figure 3. The interchain β -sheet dominated the sampling while significant sampling of intrachain sheets only occurred in the etorphine- and DAMGO-bound states and in the T279K mutation. Intrachain sheets were antiparallel, comprised of M264 to V262 hydrogen bonding with K269 to K271, while the interchain β -sheets are parallel composed of V262-R263-M264-L265, with these residues being symmetrically paired with each other in the two monomers. For example, Val262 in chain A forms backbone hydrogen bonds with Val262 in chain B. Example images of the interchain and intrachain β -sheets are shown in Fig. 3C and D respectively, with Fig. 3E showing a time frame where both the interchain and intrachain β -sheet interactions form simultaneously. In these structures the S261-V262-R263 of the second monomer forms the 3rd strand in parallel orientation with the antiparallel intrachain β -sheet.

The sampling of both helical and β -sheets structures in ICL3 is largely mutually exclusive. Simply, β -sheet formation precludes formation of the helix. This situation is necessary when β -sheet residues include K269 to K271; however in the interchain β -sheets, where residues V262 to L265 participate, the helical conformation is still not sampled even though these residues do not participate in the helices when formed. Accordingly, the simulation results indicate an equilibrium between the helical and β -sheet secondary structures in ICL3, with dimer formation favoring β -sheets and the monomer in the active conformations favoring helical states.

Summary

In summary, the present results indicate a scenario where ICL2 and ICL3 are highly dynamic. ICL2 and ICL3 connect the TM3 and TM4 and the TM5 and TM6 helices, respectively, and are known to play an important role in GPCR activation by undergoing structural rearrangements that allow for G protein binding and initiation of signal transduction.^{12, 45, 46} Deletion of the loops results in complete loss of a receptor's ability to couple to G proteins.^{47, 48} The present simulations indicate that ICL2 is in an equilibrium between helical and coiled states with the helical states favored in the active, monomer form of the receptor. The situation with ICL3 is more complex where both helical and β -sheet conformations are sampled in a mutually exclusive fashion. Sampling of helical conformations is favored in the monomeric, active conformation, an observation consistent with a study of rhodopsin signaling indicating the loop to fold into helical structures when the C-termini peptide of the G α protein binds to the receptor.⁴⁵ Alternatively, simulations of

the inactive dimer form of the μ -OR revealed sampling of β -sheet structures, including both inter- and intrachain sheets. Such conformations have not been previously observed in structures in which ICL3 is resolved^{12, 49, 50, 51} and may contribute to stabilization of the dimeric form of the μ OR. These results predict a model where receptor dimerization leads to the formation of interchain β -sheet structures that are predicted to favor the dimers thereby maintaining the OR in an inactive conformation as previously discussed. Additional studies, such as site-directed mutagenesis of residues in the ICL3, in the N-terminal region of TM6 and Asp340, are required to gain an understanding of the biological implications of this observation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Secondary structures of ICL2 and ICL3 along 5 μs dimer simulations of A) morphine-bound μ -OR; B) etorphine-bound μ -OR; C) DAMGO-bound μ -OR; D) the T279K mutant; E) β -FNA-bound μ -OR; F) naloxone-bound μ -OR. Purple corresponds to α -helix and red corresponds to β -sheet.

Figure 2.

Secondary structures of ICL2 and ICL3 along 1 μ s monomer simulations of A) morphine-bound μ -OR starting from a conformation from the dimer simulation (marked by star in Fig. S3); B) and C) etorphine-bound μ -OR starting from two different conformations from the dimer simulation (marked by diamonds in Fig. S3); D) morphine-bound μ -OR starting from the active, monomeric crystal structure; and E) apo μ -OR starting from the active, monomeric crystal structure. Purple corresponds to α -helix and red corresponds to β -sheet.

Figure 3.

(A to C) Images of conformations of ICL2 and ICL3 at 2.88 μ s of the morphine dimer MD simulation. A) The ICL2 in chain A forms a short helix spanning from Ala175 to Phe178 and (B) ICL2 in chain B forms a longer helix from Pro172 to Phe178. (C). Two ICL3 loops in the dimer form an interchain parallel β -sheet involving residues Val262, Arg263, Met 264 and Leu265 in both chains (C). (D and E) Conformation of ICL3 in the DAMGO dimer MD simulation at (D) 2.41 μ s in which two intrastrand β -sheets are present and (E) at 4.56 μ s when an intrastrand β -sheet interacts with an additional interchain strand.

Figure 4.
Sampling of the intrachain β -sheet structures in the dimeric μ OR simulations.

Figure 5.
Sampling of the interchain β -sheet structures in the dimeric μ OR simulations.