Chapter 6: Studies of α-Hydroxy Acid Incorporation *In Silico*

6.1 Introduction

Using unnatural amino acid mutagenesis, we have gained atomic-level manipulation of protein structure. Our ability to interpret the impact of our mutations is greatly aided by structural models of the neuroreceptors we study. Although recent progress in the determination of three-dimensional structures of ion channels provides a wealth of information, a full understanding of the structure-function relationship for a protein requires insight into dynamic properties as well as static structure. Combining atomic resolution structures with highly sophisticated computational approaches provides a virtual route to understanding experimental results, developing models of protein function, and making predictions that can be tested in the laboratory. Arguably, all-atom molecular dynamics simulations offer the most complete computational approach to study complex biological molecules. This approach consists of constructing an atomic model of the macromolecular system, representing the microscopic forces with a potential function, and integrating Newton's second law of motion "F=ma" to generate a trajectory. The result is essentially a movie showing the dynamic motions of the system as a function of time.

Standard molecular dynamics simulation packages offer parameters capturing the relevant physical characteristics of twenty standard amino acids. Absent, however, are parameters for unnatural amino and α-hydroxy acids. The goal of our study was therefore to develop parameters to describe backbone esters and to apply these in a molecular dynamics simulation to understand the impact of a particular ester mutation in the aromatic binding box.

6.2 Results and Discussion

6.2.1 Homology Modeling

Homology models of the extracellular domain of the mouse muscle nAChR were constructed based on the crystal structure of the acetylcholine binding protein (AChBP, PDB: 1I9B[1]) using the Modeller software package.[2, 3] This process began by aligning the sequence of the mouse muscle extracellular domain with that of the Lymnaea stagnalis AChBP.[1] After several initial alignments using TCoffee[4] produced unsatisfactory homology models, an alignment based on experimental work was used.[5] Using Lysine scanning mutagenesis, Sine et al. were able to distinguish core hydrophobic from surface hydrophilic orientations of residue side chains and use this information to align residues in AChR subunits with equivalent residues in the homologous AChBP. The Modeller program was used to generate several homology models, which were evaluated based on the following criteria: appropriate secondary structural elements, the residue positions in the highly conserved aromatic box and the F-loop, and discrete optimized protein energy (DOPE) scores.[6]

DOPE is an atomic distance-dependent statistical, or "knowledge-based," potential optimized for model assessment. This scoring potential was used to evaluate the overall quality of the initial homology models as a whole, as well as to look at the models on a residue-by-residue basis. This latter method was useful in identifying potentially problematic regions in the homology models, marked by high DOPE scores, for further optimization. Comparing the DOPE profiles of the homology models to that of the original template also proved a useful evaluation criterion (Figure 6.1).

One region that was consistently problematic was the F-loop. The F-loop in the complementary binding subunits is much longer than that in AChBP, leading to considerable variation in this region of the homology models. Numerous subsequent loop refinements were therefore used on this region. Evaluation of the position of the F-loop was based on distances between the vicinal disulfide in the C-loop and γD174/δD180, which is known to be within 9 Å based on several cross-linking studies.[7-9] The selected homology model (model 8, Figure 6.1), after loop minimization, served as the starting structure both in its unmodified form and with the backbone ester replacing the amide in residues γW55 and δW57 (see Chapter 5).

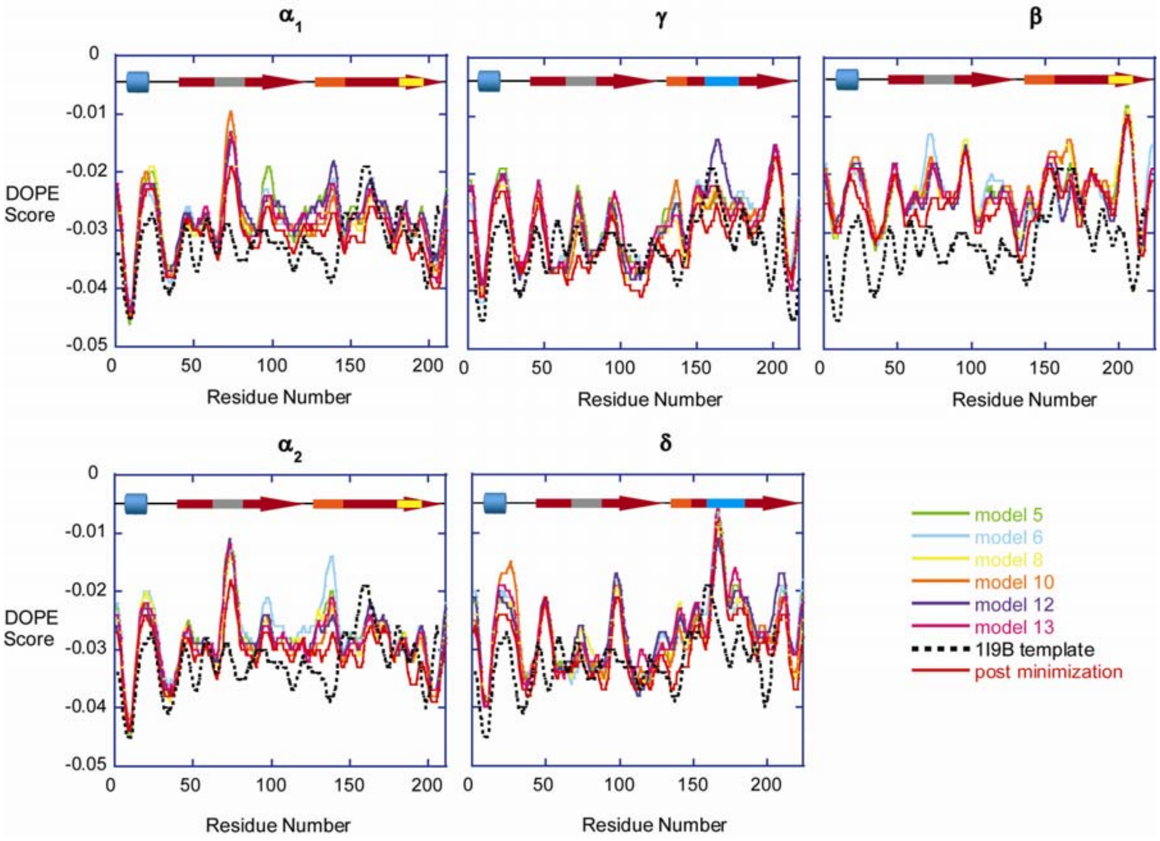


Figure 6.1: DOPE profiles for selected homology models. The protein secondary structure is highlighted above each profile. The red arrows denote β-sheets 1-6 and 7-10, respectively. The α_1 helix is designated as a blue cylinder and the MIR is shown in grey. The Cys, C-, and F-loops are represented as orange, yellow, and cyan bars, respectively.

6.2.2 Ester parameterization

To create the ester-containing protein, Pymol[10] was used to replace the backbone NH at position γW55 and δW57 with an oxygen. This structure was imported into the GROMACS simulation program and the parameters for the backbone ester were manually adjusted. The ester parameters were derived from charges in 1-lauroyl-glycerol and similar molecules in the GROMACS forcefield. In order to correctly parameterize the bond between the backbone oxygen and the i-1 carbonyl, these residues were effectively unified to create one set of parameters describing the backbone ester. These parameters were tested by comparing the behavior of a model VWL tripeptide to an analogous molecule containing a backbone ester in the middle residue. Similar behavior was observed in terms of both structure and energy, as shown in Figure 6.2.

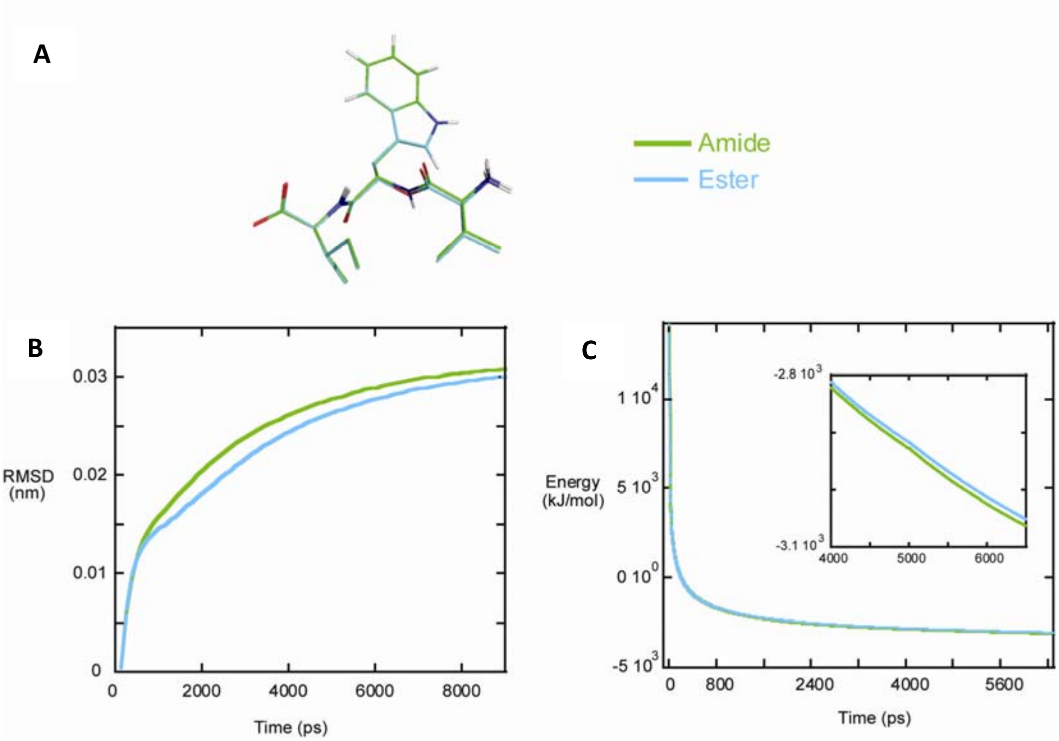


Figure 6.2: A) Structures of the amide and ester model tripeptide at the end of the minimization runs. B) The RMSD profiles of the model tripeptides. C) The energy profiles of the model tripeptides. The inset shows the energy minimization for the final 2500 ps.

6.2.3 Molecular Dynamics Simulations

The unmodified and ester-containing proteins were converted to GROMACS format and subsequently placed into a periodic box with 7 Å gaps between the protein and the box edge. Explicit solvation was added with SPC water molecules. Physiological conditions were simulation by adding sodium and chloride ions to the box at a molarity of 150 mM, with excess sodium ions to neutralize the charge of the protein. After an initial set of minimizations, unrestrained MD simulations of both the unmodified and ester-containing proteins were run to 6950 ps (see methods). The resulting trajectories were then analyzed.

6.2.3.1 Structural Analysis of MD Trajectories

Root mean-square fluctuation (RMSF) provides information about the residue mobility relative to the average structure, analogous to crystallographic B-factors. An RMSF analysis of the trajectories produced RMSF profiles and B-factors that were mapped onto the structure. This analysis shows that the ester-containing protein (<RMSF> = 0.19 nm) is moderately more mobile than the wild-type protein (<RMSF> = 0.16 nm). Examining each subunit individually reveals the greatest amount of mobility in the α subunit at the α/γ interface of the ester-containing protein. Some of the largest differences in RMSF values of individual residues between the WT and ester-modified proteins are seen in the γ-subunit, notably in the F-loop region (Figure 6.3).

The most and least mobile regions are qualitatively similar to those seen in other simulations of nAChRs,[11-15] as well as in the AChBP crystal structures.[16, 17] These regions include the MIR, the Cys-loop, the C-loop and the F-loop.

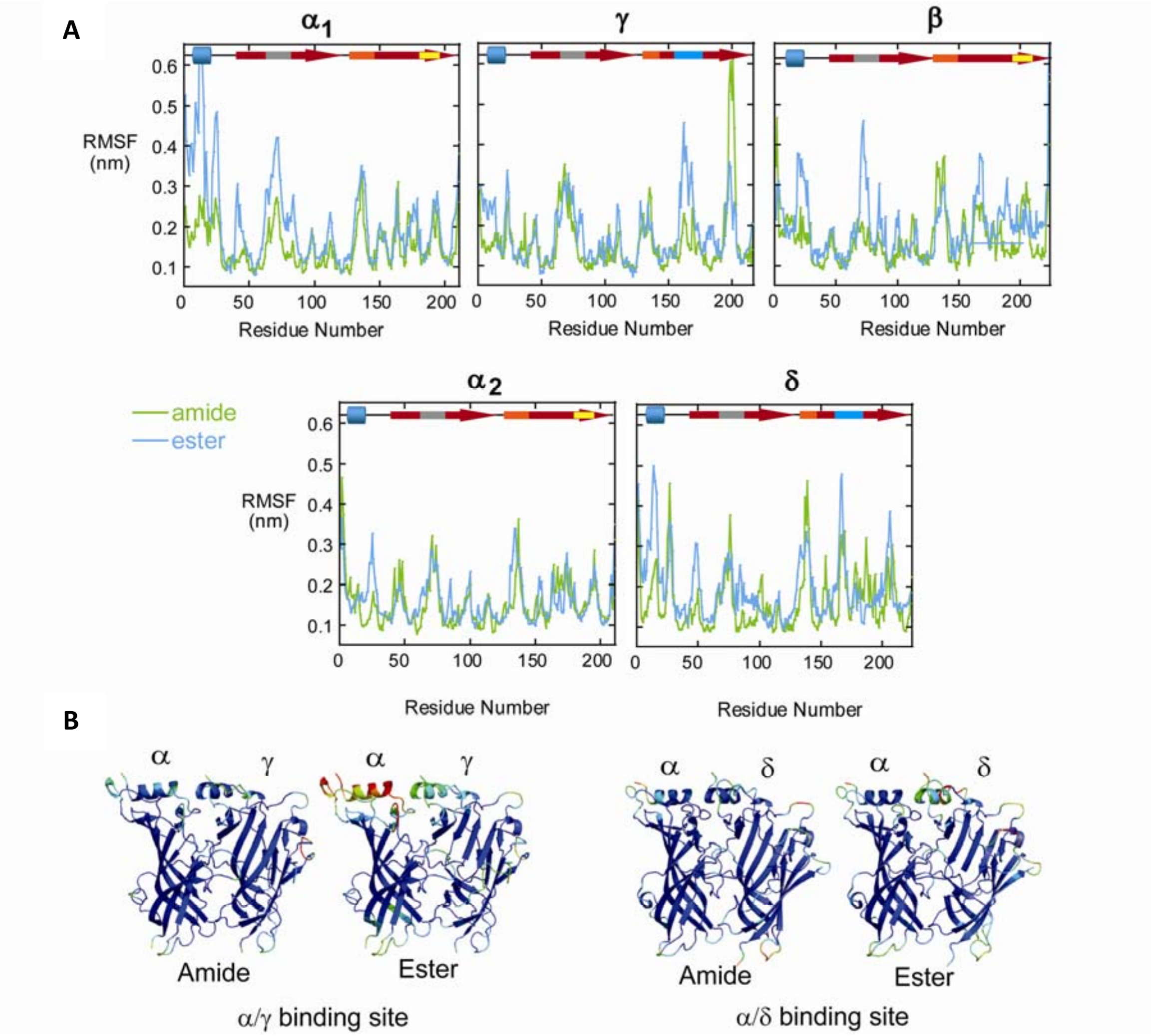


Figure 6.3: A) RMSF profiles of the WT and ester-containing proteins. The protein secondary structure is highlighted above each profile. The red arrows denote β-sheets 1-6 and 7-10, respectively. The α_1 helix is designated as a blue cylinder and the MIR is shown in grey. The Cys, C-, and F-loops are represented as orange, yellow, and cyan bars, respectively. B) The computed B-factor values color coded onto the receptor structure, with red corresponding to the most mobile region and blue corresponding to the most stable region.

The increased residue fluctuations in the ester-containing protein revealed by the RMSF analysis is further shown in the overall root mean-square deviation (RMSD) of the protein. The RMSD is used to compare the spatial deviation between structures in time and the original structure (at time = 0 ps). This can provide a sense of the overall structural stability of the simulated protein. As shown in Figure 6.4, the overall protein RMSD is higher in the ester-containing protein than in the WT protein.

Examination of the movement of the aromatic box residues reveals more divergent behavior between the ester-containing and wild-type proteins for the α/γ binding site than the α/δ binding site (Figure 6.4). Further analysis reveals additional asymmetry between the two binding sites.

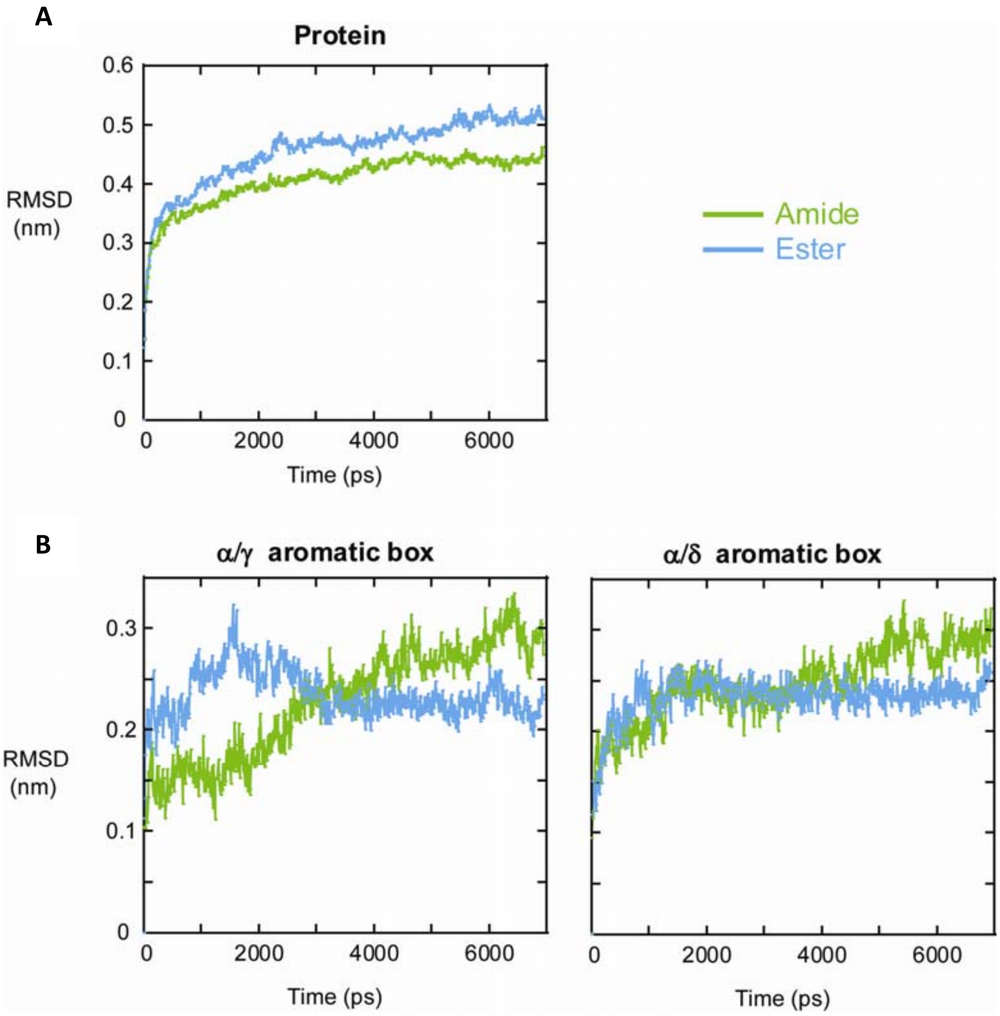


Figure 6.4: RMSD Profiles. A) The entire protein. B) The α/γ and α/δ aromatic boxes.

Looking at the side chain plane angles provides a sense of the organization of the aromatic box residues during the course of the simulation. In particular, motions of other box residues relative to the mutated box residue, γW55/δW57, were analyzed (Figure 6.5). Consistent with the box RMSDs, the plane angle fluctuations were more similar in the α/δ binding site than the α/γ binding site, although similar conformations are sampled by the wild-type and ester-containing proteins. This is perhaps best illustrated in the plane angles between αY93 and γW55. An abrupt change in plane angle from ~150° to ~50° is seen in the ester-containing protein at about 1200 ps, corresponding to the flipping of the aromatic face of the αY93 side chain relative to γW55. This side chain flipping motion is also observed in the wild-type simulation at a later point in the simulation, around 4000 ps.

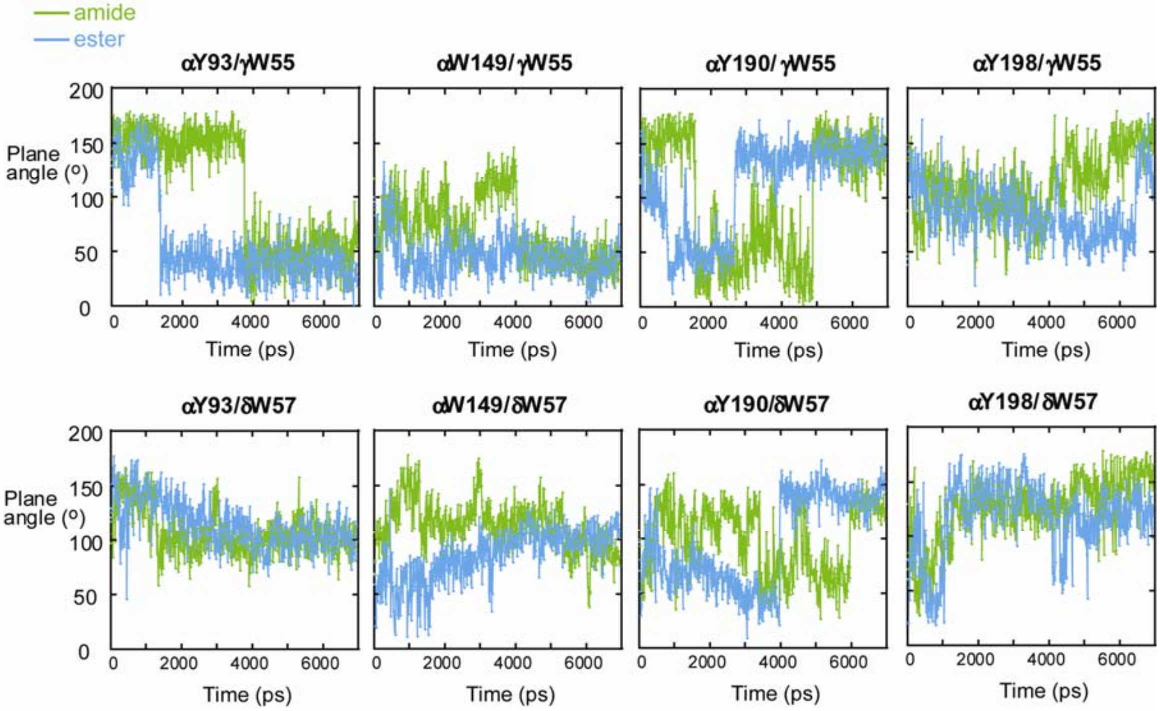


Figure 6.5: Sidechain plane angle fluctuations of the aromatic box residues.

One of the most striking differences between the unmodified and ester-containing proteins was in the behavior of the C-loop. A visual inspection of the trajectories reveals that the C-loop in the ester-containing receptor moves downward, while that of the wild-type protein more nearly remains in its original position (Figure 6.6). This motion occurs within the first several hundred picoseconds of the simulation. In addition, this motion is more pronounced in the α/γ binding site than in the α/δ binding site.

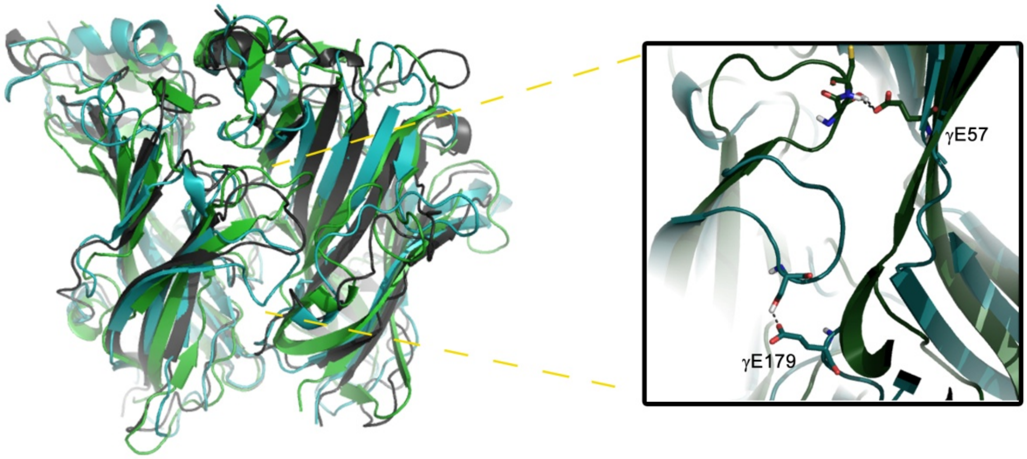


Figure 6.6: Movement of the C-loop in the α/γ interface. The grey structure is the initial structure (post-minimization, pre-MD). The hydrogen bonding between the C-loop and γE57 (WT, green) and γE179 (ester, cyan) are highlighted on the right.

An analysis of the hydrogen-bonding patterns of γW55 did not show any substantial intersubunit interactions between the C-loop and either the backbone or sidechain of this residue. A further look at all the intersubunit hydrogen bonds between the α C-loop and the adjacent subunit revealed the presence of a hydrogen bond between residues in the C-loop and γE57 in the wild-type protein that is completely absent in the ester-modified receptor. Instead, analogous C-loop residues of the ester-containing protein formed significant hydrogen bonding interactions with a different glutamate residue, γE179. As γE57 is separated from γW55 by only one residue, one reasonable hypothesis is that the backbone ester at γW55 subtly alters the side chain position of γE57, preventing the formation of the hydrogen bond between it and the C-loop. Instead, the C-loop finds an alternative hydrogen-bonding partner in γE179, thus accounting for the downward motion of the C-loop.

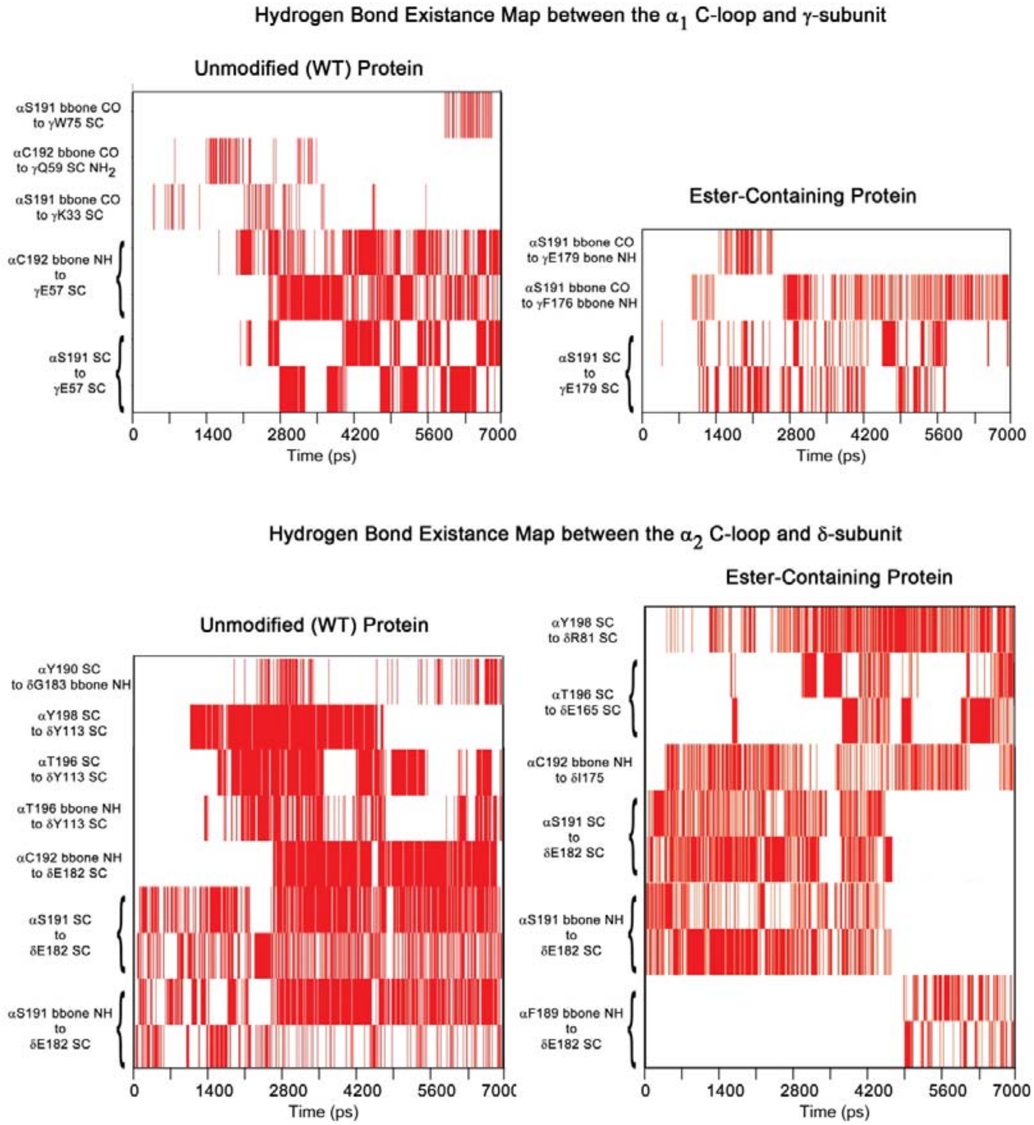


Figure 6.7: The most significantly contributing hydrogen bonds between the α C-loop and the complementary binding subunit.

The analogous residue in the δ-subunit, δE59, does not participate in hydrogen bonding interactions with the C-loop. Instead, these interactions seem to be replaced by hydrogen bonds between αS191 and the side chain of δE182, as well as between αT195 and αY198 with the side chain of δY113. Hydrogen bonds between αS191 and δE182 are also shown to be present for a significant portion of the simulation of the ester-containing protein, although those involving δY113 are largely absent. The greater overlap in the hydrogen bonding patterns seen in the α/δ binding site of the unmodified and modified proteins likely contributes to the greater similarity in C-loop movement than seen at the α/γ site.

To verify that the movement of the C-loop at the α/γ interface of the ester-containing protein is not an artifact, the first 2700 ps of the simulation were re-run. At the end of this simulation, the C-loop was in a similar position to that in the initial MD simulation (Figure 6.9). Importantly, the key hydrogen bond between αS191 in the C-loop and γE179 forms at around 2600 ps. The formation of this bond occurs later in the second simulation than in the first, where it formed within the first 1200 ps. In addition, there are some differences in the number and nature of hydrogen bonds in both the binding sites between the two simulations. Nonetheless, the movement of the C-loop is retained in the second simulation, making it likely that this motion is related to the ester modification.

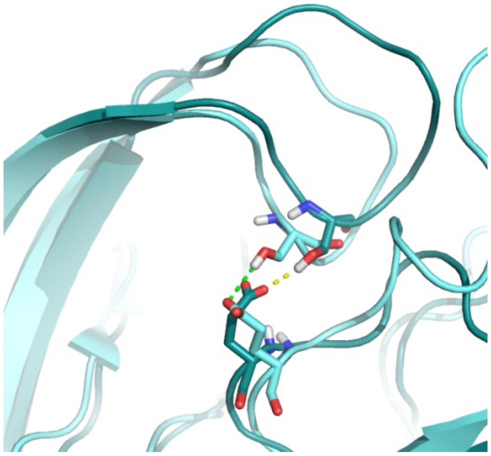


Figure 6.9: Positions of the C-loops in the first (lighter cyan) and second (darker cyan) MD simulations on the ester-containing protein at 2650 ps. Note the hydrogen bond between αS191 in the C-loop and γE179 is present in both structures.

6.2.3.2 Correlated Motion in MD Trajectories

Correlated motions between residues can be analyzed through a covariance analysis of coordinate displacements over the simulation trajectory.[18, 19] Examining correlated motions in a protein provides an understanding of which regions are strongly coupled to one another, including those that are structurally or sequentially distant. To understand the global impact of the ester mutations on protein dynamics, we performed a covariance analysis on the C^{α} atoms of the wild-type and mutant proteins using the general correlation coefficient. Using this generalized correlation measure based on mutual information (MI) allows for the complete characterization of atomic correlations without suffering from many of the artifacts arising from the use of the standard Pearson correlation coefficient.[20]

From this analysis, an increase in correlated motions is seen in the ester-containing protein. To ensure that this observation was not a result of the structural equilibration during the first 100 ps of the simulation, we evaluated the covariation matrix for the last 5 ns of the simulations only. Again, the ester-containing protein overall displayed a moderately higher degree of coupled motions (Figure 6.9). Examining the difference between the covariation matrices of the wild-type and ester-modified proteins illustrates the overall similarity in the covariation matrices. The regions where the correlated motions deviated the most between the wild-type and ester-containing proteins correspond to regions of high mobility, such as the F-loops on the α_2 and δ subunits. Notably, no substantial differences in the amount of correlated motion around the ester mutation were observed. Combined, this analysis suggests that the ester modification leads to a small change in the overall dynamics of the protein, echoing the observed overall protein RMSD similarities.

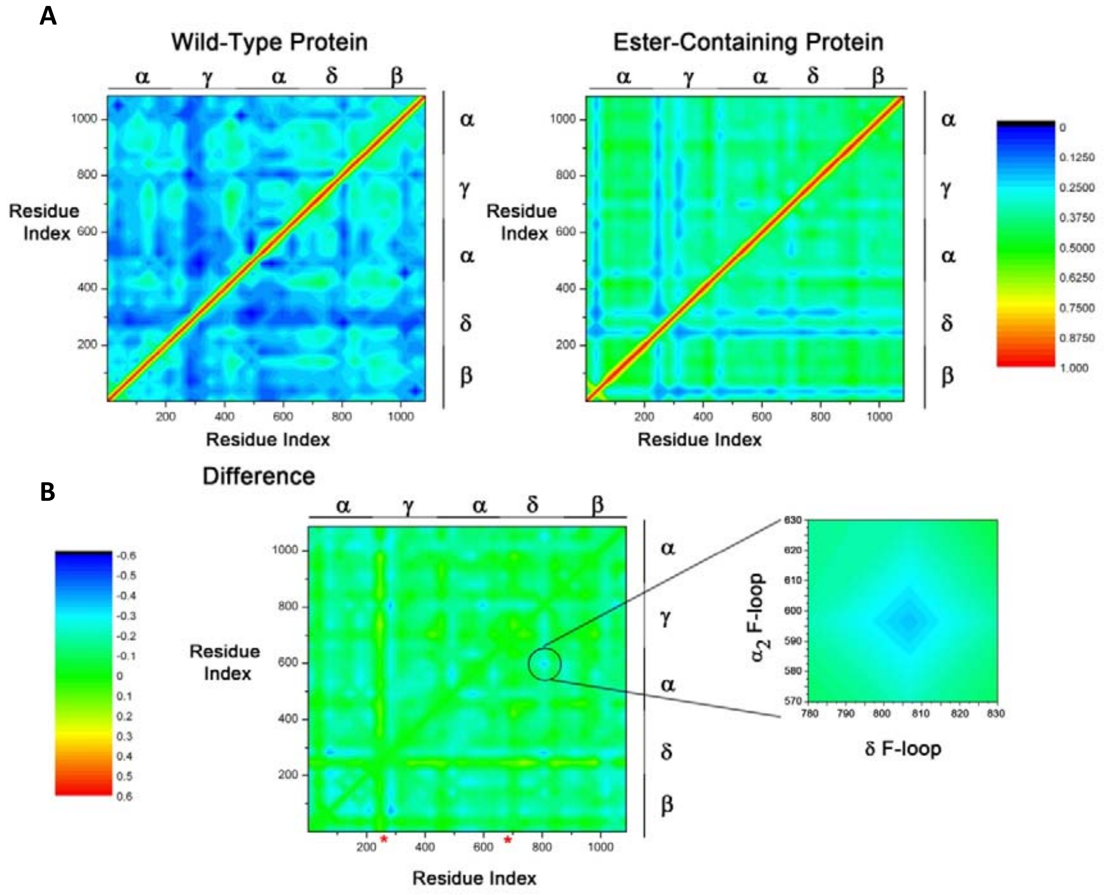


Figure 6.9: A) Correlated fluctuations of the C$^{\alpha}$ atoms in the WT and ester-containing protein. B) The differences in correlated motions between the WT and ester-containing proteins. Negative values (blue) indicate an increase in correlation motion in the ester-containing protein, while positive values (red) indicate a decrease in correlated motion. The green areas have values near zero and indicate regions of similar correlated motion in the two proteins. The red stars indicate the positions of the ester modifications.

6.3 Conclusions and Future Work

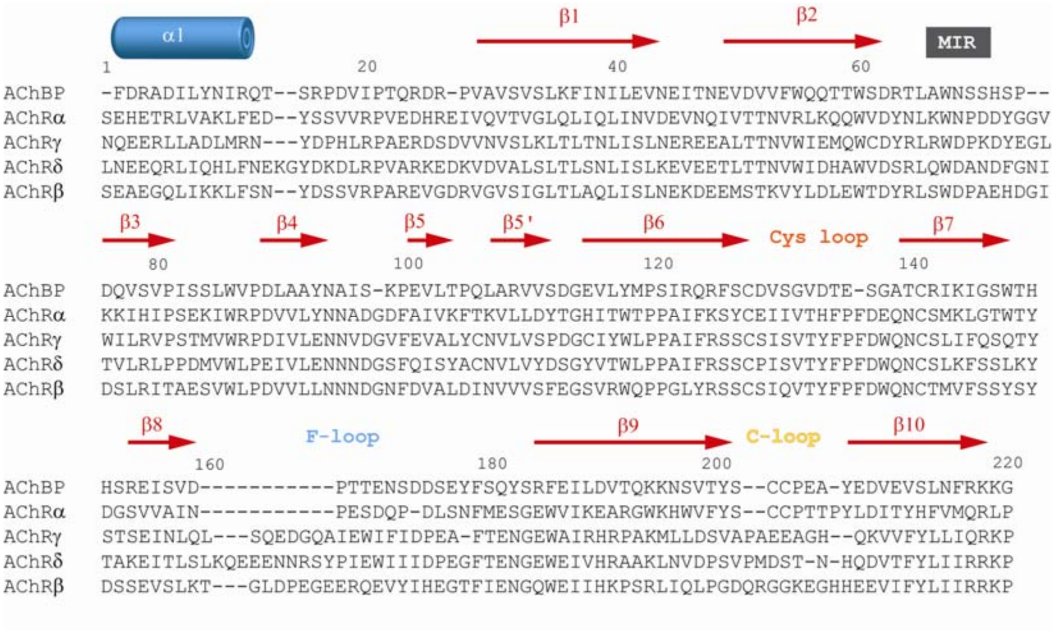
In the preliminary experiments described here, we have demonstrated that we can generate a homology model of the extracellular domain of the nAChR bearing an ester modification in two separate subunits and subject it to MD simulations. Unfortunately, the inconclusive nature of the experimental data for ester mutations at these sites makes comparison to experiment difficult in this case (see Chapter 5). However, there is still validity in the computational approach described herein for studying the effect of backbone esters in proteins. Although there are some striking differences between the unmodified and ester-bearing proteins (hydrogen-bonding patterns, loop movements, etc.), the overall results of the MD simulations show similar RMSD and RMSF patterns, as well as similar movements of the residues in the aromatic box and elsewhere around the mutated residue. Future *in silico* studies of backbone esters might include studies at well-behaved and thoroughly characterized sites in the muscle or neuronal nAChRs.

As more crystal structures of membrane receptors are being solved, our ability to model these proteins expands. The recent β_2 adrenergic GPCR structure[21] opens up the possibility of expanding our computational studies to this exciting class of membrane proteins, which are just beginning to be characterized using unnatural amino acid mutagenesis.

6.4 Materials and Methods

6.4.1 Homology Modeling Protocol

A homology model of the extracellular domain of the mouse muscle nicotinic acetylcholine receptor was constructed using Modeller 9, version 5 (http://salilab.org/modeller/), using the crystal structure of the AChBP from Lymnaea stagnalis as the template. A sequence alignment based on experimental data was used (shown in Figure 6.10). Ten three-dimensional models of the full pentamer of the mouse muscle nAChR were generated using the automodel function (see also appendix 6.1: Constructing a basic homology model using Modeller). Although six of the seven disulfide bonds were correctly calculated based on the AChBP template alone, the disulfide in the β-subunit was missing. Therefore the Cys-loop disulfides were individually specified. Each of the ten homology models were individually evaluated, as discussed in Results and Discussion. Following selection of the best initial homology model, the loop refinement feature of Modeller (loopmodel) was used to improve the position of several loop regions, again evaluated according to the criteria laid out in the Results and Discussion section.



```
AChBP   -FDRADILYNIRQT--SRPDVIPTQRDR-PVAVSVSLKFINILEVNEITNEVDVVFWQQTTWSDRTLAWNSSHSP--
AChRα   SEHETRLVAKLFED--YSSVVRPVEDHREIVQVTVGLQLIQLINVDEVNQIVTTNVRLKQQWVDYNLKWNPDDYGGV
AChRγ   NQEERLLADLMRN---YDPHLRPAERDSDVVNVSLKLTLTNLISLNEREEALTTNVWIEMQWCDYRLRWDPKDYEGL
AChRδ   LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTLSNLISLKEVEETLTTNVWIDHAWVDSRLQWDANDFGNI
AChRβ   SEAEGQLIKKLFSN--YDSSVRPAREVGDRVGVSIGLTLAQLISLNEKDEEMSTKVYLDLEWTDYRLSWDPAEHDGI

AChBP   DQVSVPISSLWVPDLAAYNAIS-KPEVLTPQLARVVSDGEVLYMPSIRQRFSCDVSGVDTE-SGATCRIKIGSWTH
AChRα   KKIHIPSEKIWRPDVVLYNNADGDFAIVKFTKVLLDYTGHITWTPPAIFKSYCEIIVTHFPFDEQNCSMKLGTWTY
AChRγ   WILRVPSTMVWRPDIVLENNVDGVFEVALYCNVLVSPDGCIYWLPPAIFRSSCSISVTYFPFDWQNCSLIFQSQTY
AChRδ   TVLRLPPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTWLPPAIFRSSCPISVTYFPFDWQNCSLKFSSLKY
AChRβ   DSLRITAESVWLPDVVLLNNNDGNFDVALDINVVVSFEGSVRWQPPGLYRSSCSIQVTYFPFDWQNCTMVFSSYSY

AChBP   HSREISVD-----------PTTENSDDSEYFSQYSRFEILDVTQKKNSVTYS--CCPEA-YEDVEVSLNFRKKG
AChRα   DGSVVAIN-----------PESDQP-DLSNFMESGEWVIKEARGWKHWVFYS--CCPTTPYLDITYHFVMQRLP
AChRγ   STSEINLQL---SQEDGQAIEWIFIDPEA-FTENGEWAIRHRPAKMLLDSVAPAEEAGH--QKVVFYLLIQRKP
AChRδ   TAKEITLSLKQEEENNRSYPIEWIIIDPEGFTENGEWEIVHRAAKLNVDPSVPMDST-N-HQDVTFYLIIRRKP
AChRβ   DSSEVSLKT---GLDPEGEERQEVYIHEGTFIENGQWEIIHKPSRLIQLPGDQRGGKEGHHEEVIFYLIIRRKP
```

Figure 6.10: The sequence alignment between Lymnaea stagnalis AChBP and the muscle nAChR subunits.

6.4.2 Construction of the ester-containing protein

The homology model for the mouse muscle nAChR extracellular domain was modified using Pymol to create the ester-containing protein. This was accomplished by manually selecting and mutating the desired backbone NH groups to oxygen. Manually, the i-1 residue and the now α-hydroxy tryptophan residue were combined into a single residue with the name "VWA." This is important because the backbone oxygen alters the amide bond between the i-1 and i residues. In order to correctly parameterize the resulting ester bond, this unified residue is used. The i-1 residue in this case was a valine, so the resulting unified dipeptide was termed "VWA." Residues in the pentamer are listed continuously. Residues 264 and 265 from the original input file were unified, as were residues 694 and 695. Since each residue can have only one C^{α}, the designation

CA was changed to CA2 for the second residue. The ester oxygen was designated OA. The resulting pdb file was then imported into GROMACS, as outlined above.

Once the GROMACS input files were initially generated (after pdb2gmx, see below), the parameters related to the backbone ester (given in appendix 6.2: backbone ester parameters) were modified in several different locations.

(1) **The .gro file** The .gro file is a fixed-column coordinate file format. Residue numbering and labeling was verified.

(2) **The local .itp files** An .itp file is a topology that is included within the system topology, and defines a topology for a single specific molecule type. It contains entries for [atoms], [bonds], [angles], [dihedrals], [impropers], and [exclusions]. Because backbone esters are not a standard part of the GROMACS program, proper parameters needed to be specified and/or verified.

> [ATOMS]: In addition to changing the appropriate VAL and TRP residues to VWA, the masses and charges were adjusted, as needed (see appendix 6.2).
>
> [BONDS], [ANGLES], [DIHEDRALS], [IMPROPERS], [EXCLUSIONS]: the bonds, angles, dihedrals, and impropers involving the backbone oxygen in the unified VWA residue needed to be specified (see appendix 6.2).

(3) **ffG43a1.rtp** This is residue topology file and must be modified as root. Each residue entry starts with a directive for the residue abbreviation (i.e., [ALA]), followed by [atoms], [bonds], [angles], [dihedrals], [impropers], and [exclusions], with the specific content dictated by the force field chosen. The new dipeptide VWA was added with parameters specified in appendix 6.2.

(4) **aminoacids.dat** Another global GROMACS file listing the amino acid types. VWA was added to this file.

(5) **ffG43a1.hdb** This is the hydrogen database, containing entries for adding missing hydrogens to residue building blocks present in the force field .rtp file.

(6) **The local .ndx file** This is an index file that contains various categories that are useful for analysis. The standard categories are: System, Protein, Protein-H, C-alpha, Backbone, MainChain, MainChain+Cb, MainChain+H, SideChain, SideChain-H, Prot-Masses, Non-Protein, SOL, NA+, CL-, Other. Because the ester containing dipeptide is a non-standard residue, it will be included in System, Protein, Protein-H, but not in C-alpha, Backbone, MainChain, MainChain+Cb, MainChain+H, SideChain, or SideChain-H. Modifying each of these categories to include the relevant atoms from "VWA" is necessary only to the extent that they will be used for analysis. Since the .ndx file often gets automatically overwritten, this file was renamed upon modification.

6.4.3 Constructing the simulation box in GROMACS

The final, loop-optimized homology model was imported into the GROMACS program using pdb2gmx, e.g.:

```
$pdb2gmx -f input_structure.pdb -o output_structure.gro -p output_topology.top -i
output_topology2.top
```

A rectangular box with dimensions 9.49600 9.36000 6.383900 was generated:

```
$editconf -f output_structure.gro -o output_structure_box.gro -d 0.7
```

The simulation box was then solvated with SPC waters:

```
$genbox -cp output_structure_box.gro -cs spc216.gro -o output_structure_box_h2o.gro -p
output_topology.top
```

A MD parameters file (parameters.mdp) was constructed for the genion run, in which ions were generated to simulate physiological conditions and neutralize the simulation box. Before any minimization or MD run is performed, it is necessary to generate a start file (.tpr). This file contains the starting structure of your simulation (coordinates and velocities), the molecular topology and all the simulation parameters. It is generated by grompp and then executed by mdrun to perform the simulation.

```
$grompp -f parameters.mdp -c output_structure_box_h2o.gro -p output_topology.top -o
run.tpr
```

For the genion run, in order to neutralize the charge in the box, it is critical to note the charge in the output. For instance, if the charge is -62 and you want to mimic a 150 mM NaCl extracellular environment in a 9 nm^3 box, then:

150 mmol/L * (9 nm)^3 * (1 cm/ 10^7 nm)^3 * 1 L/10^3 cm^3 * 1 mol/ 10^3 mmol * 6.022 x 10^{23} ions/mol = 65.9 ions

To neutralize charge, we need 62 more cations than anions, resulting in 66 cationic and 4 anionic species. This number of positively and negatively charged monovalent species is added to the box using genion:

```
$genion -s run.tpr -o output_structure_box_h2o_ion.gro -n index.ndx -g genion.log -np 66 -nn 4
-pname Na+ -nname Cl-
```

Several problems can crop up at this juncture. First, the order of the groups in the .top file must match that of the .gro file. In addition, the way the ions are specified in the .gro and .top files may need to be adjusted. For instance, it may be necessary to change Cl to CL and Na to NA in the .gro file.

At this stage, the protein in its solvated, neutral box is ready for minimization.

6.4.4 GROMACS energy minimizations

A series of seven minimization steps was performed to prepare the proteins for molecular dynamics simulations:

Minimization 1: High homology residues frozen, protein backbone strongly restrained.

Minimization 2: High homology residues frozen, protein backbone weakly restrained.

Minimization 3: Aromatic box residues frozen, protein backbone weakly restrained.

Minimization 4: No residues frozen, high homology residues strongly restrained.

Minimization 5: No residues frozen, high homology residues weakly restrained.

Minimization 6: All non-hydrogen atoms strongly restrained.

Minimization 7: Completely unrestrained.

Minimization 1:

To achieve the specified position restraints, a position restraint file is generated (restrain_backbone.itp) with the specified force constants in the X, Y, and Z directions.

```
$genpr -f output_structure_box_h2o_ion.gro -n index.ndx -o restrain_backbone.itp -fc 1000
1000 1000
```

This position restraint file is then specified in the .mdp file in the line "define = -dBACKBONE_1000" and the end of each subunit's .itp file using an if statement:

```
; Include Backbone restraint file
```

```
#ifdef BACKBONE_1000

#include "restrain_backbone.itp"

#endif
```

A new .mdp file is specified with the desired run parameters (see appendix 6.3: Minimization and Molecular Dynamics Parameter files) and compiled into an input file for the minimization run:

```
$grompp -f structure_min1.mdp -c output_structure_box_h2o_ion.gro -n index.ndx -p
output_topology.top -o structure_min1.tpr
```

The input file (.tpr) is used to initiate the minimization run, as follows:

```
$mdrun -s structure_min1.tpr -o structure_min1.trr -x structure_min1.xtc -c structure_min1.gro
-e structure_min1.edr -g structure_min1.log &
```

The file extensions designate the following:

.trr: full-precision trajectory containing coordinate, velocity, and force information

.xtc: a compressed version of the trajectory, containing only coordinate, time, and box vector information

.gro: fixed-column coordinate file format first used in the GROMOS simulation package

.edr: a portable energy file, containing all the energy terms that are saved in a simulation

.log: a log file with run information, including errors encountered

Minimization 2:

To achieve the specified position restraints, a position restraint file is generated (restrain_backbone2.itp) with the specified force constants in the X, Y, and Z directions. Note that the input coordinate file is now the output from the first minimization.

```
$genpr -f structure_min1.gro -n index.ndx -o restrain_backbone2.itp -fc 500 500 500
```

This position restraint file is then specified in the .mdp file in the line "define = -dBACKBONE_500" and the end of each subunit's .itp file using an if statement:

```
; Include Backbone restraint file
#ifdef BACKBONE_500
#include "restrain_backbone2.itp"
#endif
```

A new .mdp file is specified with the desired run parameters (see appendix 6.3: Minimization and Molecular Dynamics Parameter files) and compiled into an input file for the minimization run:

```
$grompp -f structure_min2.mdp -c structure_min1.gro -n index.ndx -p output_topology.top -o
structure_min2.tpr
```

The input file (.tpr) is used to initiate the minimization run, as follows:

```
$mdrun -s structure_min2.tpr -o structure_min2.trr -x structure_min2.xtc -c structure_min2.gro
-e structure_min2.edr -g structure_min2.log &
```

Minimization 3:

No additional position restraint files were needed for this minimization. A new .mdp file is specified with the desired run parameters (see appendix 6.3: Minimization and Molecular Dynamics Parameter files) and compiled into an input file for the minimization run:

```
$grompp -f structure_min3.mdp -c structure_min2.gro -n index.ndx -p output_topology.top -o
structure_min3.tpr
```

The input file (.tpr) is used to initiate the minimization run, as follows:

```
$mdrun -s structure_min3.tpr -o structure_min3.trr -x structure_min3.xtc -c structure_min3.gro
-e structure_min3.edr -g structure_min3.log &
```

Minimization 4:

To achieve the specified position restraints, a position restraint file is generated (Homology_1000.itp) with the specified force constants in the X, Y, and Z directions. Note that the input coordinate file is now the output from the third minimization.

```
$genpr -f structure_min2.gro -n index.ndx -o Homology_1000.itp -fc 1000 1000 1000
```

This position restraint file is then specified in the .mdp file in the line "define = -dHomology_1000" and the end of each subunit's .itp file using an if statement:

```
; Include Backbone restraint file
#ifdef Homology_1000
#include "Homology_1000.itp"
#endif
```

A new .mdp file is specified with the desired run parameters (see appendix 6.3: Minimization and Molecular Dynamics Parameter files) and compiled into an input file for the minimization run:

```
$grompp -f structure_min4.mdp -c structure_min3.gro -n index.ndx -p output_topology.top -o
structure_min4.tpr
```

The input file (.tpr) is used to initiate the minimization run, as follows:

```
$mdrun -s structure_min4.tpr -o structure_min4.trr -x structure_min4.xtc -c structure_min4.gro -e structure_min4.edr -g structure_min4.log &
```

Minimization 5:

To achieve the specified position restraints, a position restraint file is generated (Homology_500.itp) with the specified force constants in the X, Y, and Z directions. Note that the input coordinate file is now the output from the fourth minimization.

```
$genpr -f structure_min2.gro -n index.ndx -o Homology_500.itp -fc 500 500 500
```

This position restraint file is then specified in the .mdp file in the line "define = -dHomology_500" and the end of each subunit's .itp file using an if statement:

```
; Include Backbone restraint file
#ifdef Homology_500
#include "Homology_500.itp"
#endif
```

A new .mdp file is specified with the desired run parameters (see appendix 6.3: Minimization and Molecular Dynamics Parameter files) and compiled into an input file for the minimization run:

```
$grompp -f structure_min5.mdp -c structure_min4.gro -n index.ndx -p output_topology.top -o structure_min5.tpr
```

The input file (.tpr) is used to initiate the minimization run, as follows:

```
$mdrun -s structure_min5.tpr -o structure_min5.trr -x structure_min5.xtc -c structure_min5.gro -e structure_min5.edr -g structure_min5.log &
```

Minimization 6:

To achieve the specified position restraints, a position restraint file is generated (nonH_1000.itp) with the specified force constants in the X, Y, and Z directions. Note that the input coordinate file is now the output from the fifth minimization.

```
$genpr -f structure_min2.gro -n index.ndx -o nonH_1000.itp -fc 1000 1000 1000
```

This position restraint file is then specified in the .mdp file in the line "define = -dnonH_1000" and the end of each subunit's .itp file using an if statement:

```
; Include Backbone restraint file
#ifdef nonH_1000
#include " nonH_1000.itp"
#endif
```

A new .mdp file is specified with the desired run parameters (see appendix 6.3: Minimization and Molecular Dynamics Parameter files) and compiled into an input file for the minimization run:

```
$grompp -f structure_min6.mdp -c structure_min5.gro -n index.ndx -p output_topology.top -o
structure_min6.tpr
```

The input file (.tpr) is used to initiate the minimization run, as follows:

```
$mdrun -s structure_min6.tpr -o structure_min6.trr -x structure_min6.xtc -c structure_min6.gro
-e structure_min6.edr -g structure_min6.log &
```

Minimization 7:

The final minimization had no position restraints. A new .mdp file is specified with the desired run parameters (see appendix 3: Minimization and Molecular Dynamics Parameter files) and compiled into an input file for the minimization run:

```
$grompp -f structure_min7.mdp -c structure_min6.gro -n index.ndx -p output_topology.top -o structure_min7.tpr
```

The input file (.tpr) is used to initiate the minimization run, as follows:

```
$mdrun -s structure_min7.tpr -o structure_min7.trr -x structure_min7.xtc -c structure_min7.gro -e structure_min7.edr -g structure_min7.log &
```

Upon completion of all the minimization steps, the trajectories were concatenated and the energy and RMSD profiles of these runs were examined, along with the final structure (Figure 6.4).

```
trjcat -f structure_min1.trr structure_min2.trr structure_min3.trr structure_min4.trr structure_min5.trr structure_min6.trr structure_min7.trr -o concat_min1_7.xtc -n index.ndx

g_rms -s structure_min1.tpr -f concat_min1_7.xtc -o concat_min1_7_rms.xvg

g_energy -f structure_min1.edr -o structure_min1_nrg.xvg (and so on for each minimization)

editconf -f structure_min7.gro -n index.ndx -o structure_min7.pdb
```

6.4.5 GROMACS Molecular Dynamics Simulations

Once it was confirmed that the energy and structure from the minimizations were sensible (i.e. no dramatic deviations from the initial homology model and no anomalous energy fluctuations, see Figure 6.4), a series of molecular dynamics runs were initiated.

The MD runs were begun at 0 K and the temperature was gradually increased to 310 K with a linear annealing function over the first 25 ps (see appendix 6.3: Minimization and Molecular Dynamics Parameter files). The protein was strongly constrained during this warm-up phase and then the restraints were relaxed over the subsequent 125 ps. After this point the simulations proceeded unrestrained.

MD1: 50 ps. Annealing from 0 to 310 K over the first 25 ps, held at 310 K for the remaining 25 ps. Protein strongly restrained.

MD2: 50 ps. Backbone strongly restrained.

MD3: 50 ps. Backbone weakly restrained.

MD4-11: 850 ps each. No restraints.

MD1:

Position restraints were fulfilled and specified in the appropriate places, as was done in the minimization runs.

```
$genpr -f structure_min7.gro -n index.ndx -o restrain_protein.itp -fc 1000 1000 1000
```

MD input file was compiled:

```
$grompp -f structure_md1.mdp -c structure_min7.gro -n index.ndx -p  output_topology.top -o
structure_md1.tpr
```

MD run initiated:

```
$mdrun -s structure_md1.tpr -o structure_md1.trr -x structure_md1.xtc -c
structure_md1.gro -e structure_md1.edr -g structure_md1.log &
```

MD2-11:

Position restraints were fulfilled and specified in the appropriate places, as was done in the minimization runs. No additional positional restraint files were needed, because the backbone constraint files had already been generated during the minimizations. The time designated at the start of the run, tint, is changed in each of these MD runs to reflect the simulation time of the previous MD runs (e.g. MD2 begins at 50 ps instead of 0 ps, where MD1 ended).

MD input file was compiled:

```
$grompp -f structure_md2.mdp -c structure_md1.gro -n index.ndx -p output_topology.top -o
structure_md2.tpr
```

MD run initiated:

```
$mdrun -s structure_md2.tpr -o structure_md2.trr -x structure_md2.xtc -c
structure_md2.gro -e structure_md2.edr -g structure_md2.log &
```

6.4.5 Analysis

RMSD values were calculated using g_rms. RMSF values were calculated on the concatenated trajectories using g_rmsf.

Side chain plane angles were calculated using g_sgangle. Index groups were defined for each side chain. Tryptophan side chains were defined by CG, CZ3, and NE atoms. Tyrosine side chains were defined by the CG, CE1, and CE2 atoms. g_sgangle was used with the option -noone to calculated interplane angles between two residues.

Hydrogen bonding patterns were evaluated using g_hbond. Non-overlapping groups were created in the index file for this analysis. The resulting .xpm file was converted to an .eps format using xpm2ps.

The correlation matrices were calculated on the protein α-carbons using g_correlation, downloaded from http://www.mpibpc.mpg.de/groups/grubmueller/olange/gencorr.html and installed in GROMACS. MATLAB[22] was used to read the *.dat output generated by g_correlation and produce the matrix of correlation coefficients. This matrix was written to Microsoft Excel before being exported and plotted using the Origin software program.[23]

6.5 References

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Appendix 6.1: Constructing a basic homology model using Modeller

Step 1: Construct a good target-template alignment.

The target sequence- sequence of desired homology model

The template sequence- sequence of the existing structure (ie AChBP)

The quality of your sequence alignment will strongly influence the quality of your homology model. I recommend using a sequence alignment that is based partially on experimental results (when possible).

The format of your sequence alignment should be as is shown in the file mm_nAChR.ali

-The first line of each sequence entry specifies the protein code after the >P1; line identified.

-The second line contains information necessary to extract the atomic coordinates of the segment from the original PDB coordinate set. Fields are separated by colon characters.

-**Field 1**: specification of whether or not the 3D structure is available. For our purposes, this will be either structureX (for x-ray structure of template) or sequence (for the target).
-**Field 2**: The PDB code
-**Field 3-6**: the residue and chain identifiers for the first and last residue of the sequence given in the subsequent lines. Note in the example file, this field is **1:A:+1025:E:** for the template, specifying that the first residue is 1 in chain A and the last is 1025 in chain E. In the target sequence, these fields are unnecessary.
-**Field 7**: Protein name (optional)
-**Field 8**: Source of protein (optional)
-**Field 9**: Resolution (optional)
-**Field 10**: R-factor (optional)

-The subsequent lines have the aligned sequence in them**.** Chain breaks are indicated by " / ". **If you are using AChBP for a template, this is the only part of this file you will need to change.**

Step 2: Build your model using model.py

Once the target-template alignment is ready, MODELLER calculates a 3D model of the target completely automatically, using the automodel class. The model.py script will generate 5 similar models of your target based on the template. There are several features of this script that you should be aware of.

- The **special_restraints** (below) part of the script constrains the C_{α}s in chains A and C to be the same. This was done because there are 2 alpha subunits in the muscle nicotinic receptor. It is not strictly necessary to do this.

```
def special_restraints(self, aln):
                s1 = selection(self.chains['A']).only_atom_types('CA')
                s2 = selection(self.chains['C']).only_atom_types('CA')
                self.restraints.symmetry.append(symmetry(s1, s2, 1.0))
                rsr = self.restraints
                at = self.atoms
```

-The special_patches (below) part of the script forces a disulfide bond between the specified residues (in the specified chain) and to specify secondary structural elements. Neither of these things is strictly necessary, however, it is sometimes handy to be able to do. # is used to "comment out" a line, i.e., if you put it before a line, the program will ignore that line.

```
def special_patches(self, aln):
        # A disulfide between residues 128 and 142, ie the Cys-loop:
        #self.patch(residue_type='DISU', residues=(self.residues['128:A'], self.residues['142:A']))
        #self.patch(residue_type='DISU', residues=(self.residues['338:B'], self.residues['352:B']))
        #self.patch(residue_type='DISU', residues=(self.residues['557:C'], self.residues['571:C']))
        #self.patch(residue_type='DISU', residues=(self.residues['770:D'], self.residues['784:D']))
        self.patch(residue_type='DISU', residues=(self.residues['993:E'], self.residues['1007:E']))
#residues 1 thru 10 of chain A and B should be an alpha helix
        #rsr.add(secondary_structure.alpha(self.residue_range('1:A', '10:A')))
        #rsr.add(secondary_structure.alpha(self.residue_range('483:C', '499:C')))
        #rsr.add(secondary_structure.alpha(self.residue_range('239:B', '251:B')))
```

-The last several lines of the script are crucial:

```
a = MyModel(env, alnfile='mm_nAChR-mult.ali',

                 knowns=('1I9B'), sequence='mm_nAChR',
                 assess_methods=(assess.DOPE, assess.GA341))

a.starting_model = 1
a.ending_model = 5
a.make()
```

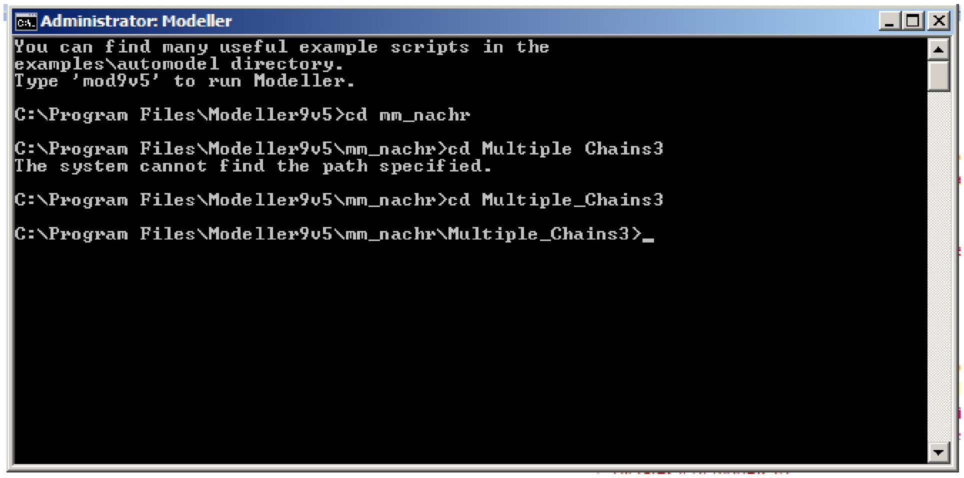
The name, as specified in your .ali file, of the template you are using.

The name, as specified in your .ali file, of the target sequence

Assessment methods provide a quick way of evaluating various models relative to one another.

Dictates # of models to generate.

Once you have model.py specified the way you want, you should open the Modeller program from the start menu of your computer. This will give you a DOS command prompt window. Now you need to be in the proper directory. To change directories, type cd followed by the directory you want (use tab to auto complete the folder name). Your default start directory will be in the Modeller9v5 directory in your program files directory:



```
You can find many useful example scripts in the
examples\automodel directory.
Type 'mod9v5' to run Modeller.

C:\Program Files\Modeller9v5>cd mm_nachr

C:\Program Files\Modeller9v5\mm_nachr>cd Multiple Chains3
The system cannot find the path specified.

C:\Program Files\Modeller9v5\mm_nachr>cd Multiple_Chains3

C:\Program Files\Modeller9v5\mm_nachr\Multiple_Chains3>_
```

Once you are in the proper directory, type: **mod9v5 model.py**, which will run the script for you. It will take several minutes for the script to finish.

Step 3: Evaluate your model

Step 3A: Since we included assess_methods in our model.py script, the bottom of the output log file (named model.log) will have a summary of the produced models, listing both DOPE and GA341 scores. These provide a quick and dirty way to assess which model is best.

-The DOPE (Discrete Optimized Protein Energy) is the more reliable of the two. The lowest (most negative) value is the best.

-GA341 is another method. Here 0.0 is worst and 1.0 is best.

Step 3B: Since the assessment scores mentioned above are for the entire protein, you might want to know which residues/regions of the model are the "worst." To do this, you need to run an assessment. The script for this is called evaluate_model.py. The output file will be *.profile that you specify at the end of the script (below- mm_nAChR_min7.profile).

```
s.assess_dope(output='ENERGY_PROFILE NO_REPORT', file='mm_nAChR_min7.profile',
              normalize_profile=True, smoothing_window=15)
```

-To run the script, type: mod9v5 evaluate_model.py

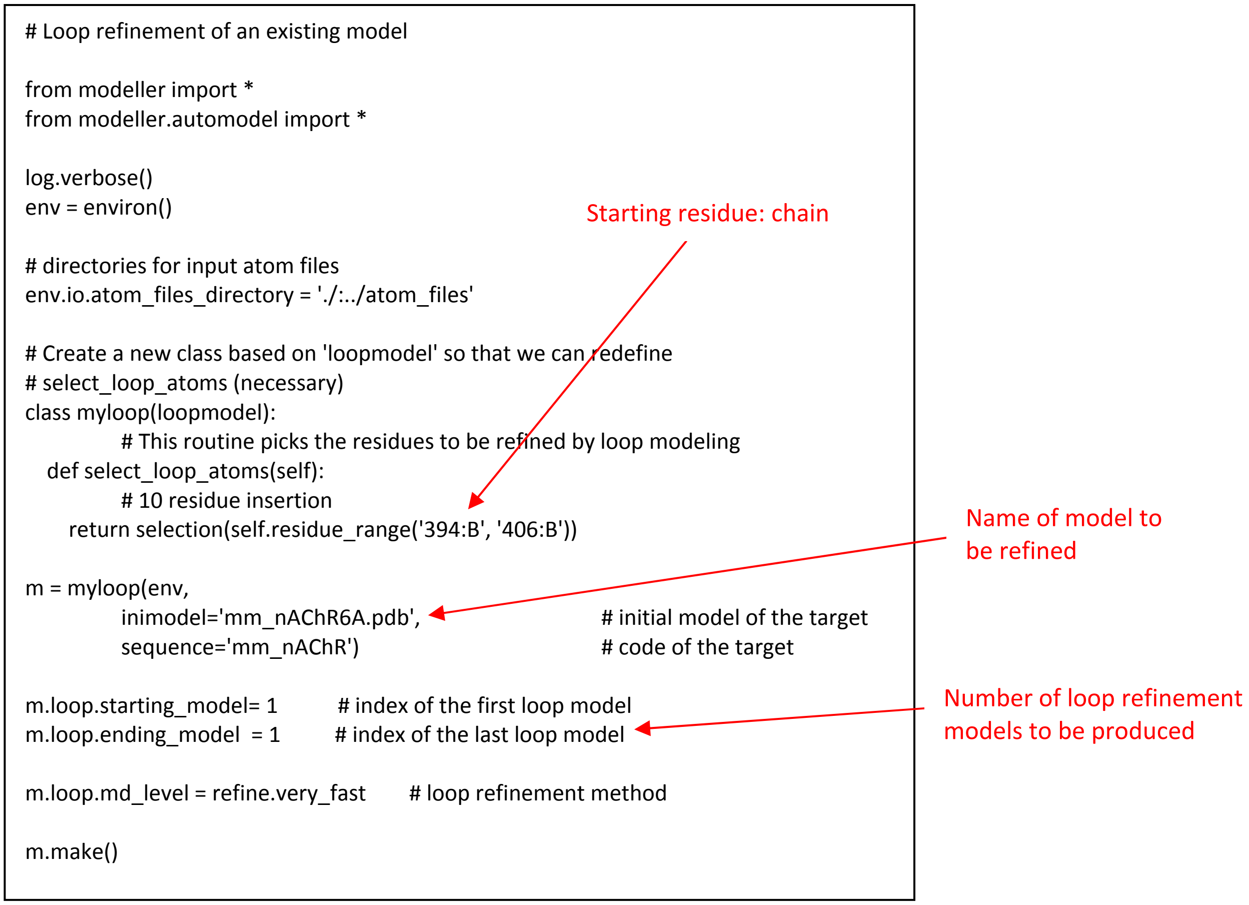
-You will want to paste the output contained in the *.profile file into excel, using the text-to-column delimited option. From there you can plot the DOPE score on a residue by residue basis. This is convenient because you are then able to hone in on potential problem spots in your homology model when looking at them using whatever visualization program you prefer.

Step 4: Refine loops (optional)

Depending on how your model looks, you may decide to refine some of the loops in your structure. For example, Loop F is much shorter in AChBP than it is in the d and g subunits of the muscle receptor, which lead to some odd loop conformations in my original homology models. These could be fixed using the loop_refine.py script.

-You will want to specify the amino acids that define the loop, as well as which homology model you want to refine (remember in step 3 we generated 5 separate models). You will also get to specify the number of independent loop refinements you want the program to run.

-To run, type: mod9v5 loop_refine.py



```
# Loop refinement of an existing model

from modeller import *
from modeller.automodel import *

log.verbose()
env = environ()

# directories for input atom files
env.io.atom_files_directory = './:../atom_files'

# Create a new class based on 'loopmodel' so that we can redefine
# select_loop_atoms (necessary)
class myloop(loopmodel):
            # This routine picks the residues to be refined by loop modeling
   def select_loop_atoms(self):
            # 10 residue insertion
      return selection(self.residue_range('394:B', '406:B'))

m = myloop(env,
            inimodel='mm_nAChR6A.pdb',                      # initial model of the target
            sequence='mm_nAChR')                            # code of the target

m.loop.starting_model= 1        # index of the first loop model
m.loop.ending_model  = 1        # index of the last loop model

m.loop.md_level = refine.very_fast       # loop refinement method

m.make()
```

Starting residue: chain

Name of model to be refined

Number of loop refinement models to be produced

Appendix 6.2: Backbone Ester Parameters for GROMACS

```
[ VWA ]
[ atoms ]
N    N    -0.28000    0
H    H     0.28000    0
CA   CH1    0.00000    1
CB   CH1    0.00000    1
CG1  CH3    0.00000    1
CG2  CH3    0.00000    1
C    C      0.270    2
O    O     -0.190    2
OA   OA   -0.18000    0
CA2  CH1    0.10000    1
CB2  CH2    0.00000    1
CG3   C   -0.14000    2
CD1   C   -0.10000    2
HD1   HC    0.10000    2
CD2   C    0.00000    2
NE1   NR   -0.05000    2
HE1   H    0.19000    2
CE2   C    0.00000    2
CE3   C   -0.10000    3
HE3   HC    0.10000    3
CZ2   C   -0.10000    4
HZ2   HC    0.10000    4
CZ3   C   -0.10000    5
HZ3   HC    0.10000    5
CH2   C   -0.10000    6
HH2   HC    0.10000    6
C2    C      0.380    7
O2    O     -0.380    7
```

```
[ bonds ]
N    H    gb_2
N    CA   gb_20
CA    C   gb_26
C    O   gb_4
C    OA   gb_17
CA   CB   gb_26
CB   CG1   gb_26
CB   CG2   gb_26
OA   CA2   gb_17
CA2   C2   gb_26
C2   O2   gb_4
C2   +N   gb_9
CA2  CB2   gb_26
CB2  CG3   gb_26
CG3  CD1   gb_9
CG3  CD2   gb_15
CD1  HD1   gb_3
CD1  NE1   gb_9
CD2  CE2   gb_15
CD2  CE3   gb_15
NE1  HE1   gb_2
NE1  CE2   gb_9
CE2  CZ2   gb_15
CE3  HE3   gb_3
CE3  CZ3   gb_15
CZ2  HZ2   gb_3
CZ2  CH2   gb_15
CZ3  HZ3   gb_3
CZ3  CH2   gb_15
CH2  HH2   gb_3
```

```
[ exclusions ]
;  ai    aj
CB2   HD1
CB2   NE1
CB2   CE2
CB2   CE3
CG3   HE1
CG3   HE3
CG3   CZ2
CG3   CZ3
CD1   CE3
CD1   CZ2
HD1   CD2
HD1   HE1
HD1   CE2
CD2   HE1
CD2   HZ2
CD2   HZ3
CD2   CH2
NE1   CE3
NE1   HZ2
NE1   CH2
HE1   CZ2
CE2   HE3
CE2   CZ3
CE2   HH2
CE3   CZ2
CE3   HH2
HE3   HZ3
HE3   CH2
CZ2   HZ3
HZ2   CZ3
HZ2   HH2
HZ3   HH2
```

```
[ angles ]
;  ai    aj    ak   gromos type
-C    N    H    ga_31
H    N    CA    ga_17
-C    N    CA    ga_30
N    CA    C    ga_12
CA    C    OA    ga_18
CA    C    O    ga_29
O    C    OA    ga_32
N    CA    CB    ga_12
C    CA    CB    ga_12
CA    CB    CG1    ga_14
CA    CB    CG2    ga_14
CG1    CB    CG2    ga_14
C    OA    CA2    ga_9
OA    CA2    C2    ga_12
CA2    C2    +N    ga_18
CA2    C2    O2    ga_29
O2    C2    +N    ga_32
OA    CA2    CB2    ga_12
C2    CA2    CB2    ga_12
CA2    CB2    CG3    ga_14
CB2    CG3    CD1    ga_36
CB2    CG3    CD2    ga_36
CD1    CG3    CD2    ga_6
CG3    CD1    HD1    ga_35
HD1    CD1    NE1    ga_35
CG3    CD1    NE1    ga_6
CG3    CD2    CE2    ga_6
CD1    NE1    CE2    ga_6
CD1    NE1    HE1    ga_35
HE1    NE1    CE2    ga_35
NE1    CE2    CD2    ga_6
CG3    CD2    CE3    ga_38
NE1    CE2    CZ2    ga_38
CD2    CE2    CZ2    ga_26
CE2    CD2    CE3    ga_26
CD2    CE3    HE3    ga_24
HE3    CE3    CZ3    ga_24
CD2    CE3    CZ3    ga_26
CE2    CZ2    HZ2    ga_24
HZ2    CZ2    CH2    ga_24
CE2    CZ2    CH2    ga_26
CE3    CZ3    HZ3    ga_24
HZ3    CZ3    CH2    ga_24
CE3    CZ3    CH2    ga_26
CZ2    CH2    HH2    ga_24
HH2    CH2    CZ3    ga_24
CZ2    CH2    CZ3    ga_26
```

```
[ impropers ]
; ai aj ak al gromos type
N -C CA H gi_1
C CA OA O gi_1
CA N C CB gi_2
CB CG2 CG1 CA gi_2
C2 CA2 +N O2 gi_1
CA2 OA C2 CB2 gi_2
CG3 CD1 CD2 CB2 gi_1
CD2 CG3 CD1 NE1 gi_1
CD1 CG3 CD2 CE2 gi_1
CG3 CD1 NE1 CE2 gi_1
CG3 CD2 CE2 NE1 gi_1
CD1 NE1 CE2 CD2 gi_1
CD1 CG3 NE1 HD1 gi_1
NE1 CD1 CE2 HE1 gi_1
CD2 CE2 CE3 CG3 gi_1
CE2 CD2 CZ2 NE1 gi_1
CE3 CD2 CE2 CZ2 gi_1
CD2 CE2 CZ2 CH2 gi_1
CE2 CD2 CE3 CZ3 gi_1
CE2 CZ2 CH2 CZ3 gi_1
CD2 CE3 CZ3 CH2 gi_1
CE3 CZ3 CH2 CZ2 gi_1
CE3 CD2 CZ3 HE3 gi_1
CZ2 CE2 CH2 HZ2 gi_1
CZ3 CE3 CH2 HZ3 gi_1
CH2 CZ2 CZ3 HH2 gi_1

[ dihedrals ]
; ai aj ak al gromos type
-CA -C N CA gd_4
-C N CA C gd_19
N CA C OA gd_20
N CA CB CG1 gd_17
CA C OA CA2 gd_3
C OA CA2 C2 gd_12
OA CA2 C2 +N gd_17
OA CA2 CB2 CG3 gd_20
CA2 CB2 CG3 CD2 gd_20
```

Appendix 6.3: Minimization and Molecular Dynamics Parameter files

WT and ester-containing files are equivalent with the sole exception of the title.

Minimization 1: mm_nachr_min1.mdp, wah_nachr_min1.mdp

```
title                     = wah_nachr_min1
cpp                       = /lib/cpp
define                    = -dBACKBONE_1000
integrator                = steep
emstep                    = 0.01
tinit                     = 0.0
nsteps                    = 5000
nstlog                    = 10
nstlist                   = 10
ns_type                   = grid
rlist                     = 0.8
coulombtype               = PME
fourierspacing            = 0.10
pme_order                 = 4
ewald_rtol                = 1e-5
epsilon_r                 = 80
rcoulomb                  = 0.8
rvdw                      = 0.8
freeze_grps               = high_homology
freeze_dim                = y y y
```

Minimization 2: mm_nachr_min2.mdp, wah_nachr_min2.mdp

```
title                     = wah_nachr_min2
cpp                       = /lib/cpp
define                    = -dBACKBONE_500
integrator                = steep
emstep                    = 0.01
tinit                     = 0.0
nsteps                    = 5000
nstlog                    = 10
nstlist                   = 10
ns_type                   = grid
rlist                     = 0.8
coulombtype               = PME
fourierspacing            = 0.10
pme_order                 = 4
ewald_rtol                = 1e-5
epsilon_r                 = 80
rcoulomb                  = 0.8
rvdw                      = 0.8
freeze_grps               = high_homology
freeze_dim                = y y y
```

Minimization 3: mm_nachr_min3.mdp, wah_nachr_min3.mdp

```
title                     = wah_nachr_min3
cpp                       = /lib/cpp
define                    = -dBACKBONE_500
integrator                = steep
emstep                    = 0.01
tinit                     = 0.0
nsteps                    = 5000
nstlog                    = 10
nstlist                   = 10
```

```
ns_type                  = grid
rlist                    = 0.8
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
epsilon_r                = 80
rcoulomb                 = 0.8
rvdw                     = 0.8
freeze_grps              = boxes
freeze_dim               = y y y
```

Minimization 4: mm_nachr_min4.mdp, wah_nachr_min4.mdp

```
title                    = wah_nachr_min4
cpp                      = /lib/cpp
define                   = -dHomology_1000
integrator               = steep
emstep                   = 0.01
tinit                    = 0.0
nsteps                   = 10000
nstlog                   = 10
nstlist                  = 10
ns_type                  = grid
rlist                    = 0.8
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
epsilon_r                = 80
rcoulomb                 = 0.8
rvdw                     = 0.8
```

Minimization 5: mm_nachr_min5.mdp, wah_nachr_min5.mdp

```
title                    = wah_nachr_min5
cpp                      = /lib/cpp
define                   = -dHomology_500
integrator               = steep
emstep                   = 0.01
tinit                    = 0.0
nsteps                   = 6000
nstlog                   = 10
nstlist                  = 10
ns_type                  = grid
rlist                    = 0.8
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
epsilon_r                = 80
rcoulomb                 = 0.8
rvdw                     = 0.8
```

Minimization 6: mm_nachr_min6.mdp, wah_nachr_min6.mdp

```
title                    = wah_nachr_min6
cpp                      = /lib/cpp
define                   = -dnonH_1000
integrator               = steep
```

```
emstep                   = 0.01
tinit                    = 0.0
nsteps                   = 6000
nstlog                   = 10
nstlist                  = 10
ns_type                  = grid
rlist                    = 0.8
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
epsilon_r                = 80
rcoulomb                 = 0.8
rvdw                     = 0.8
```

Minimization 7: mm_nachr_min7.mdp, wah_nachr_min7.mdp

```
title                    = wah_nachr_min7
cpp                      = /lib/cpp
define                   =
integrator               = steep
emstep                   = 0.01
tinit                    = 0.0
nsteps                   = 10000
nstlog                   = 10
nstlist                  = 10
ns_type                  = grid
rlist                    = 0.8
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
epsilon_r                = 80
rcoulomb                 = 0.8
rvdw                     = 0.8
```

MD Run 1: mm_nachr_md1.mdp, wah_nachr_md1.mdp

```
title                    = wah_nachr_md1
cpp                      = /lib/cpp
define                   = -dProtein_1000
integrator               = md
dt                           = 0.002
tinit                    = 0.0
nsteps                   = 25000
nstxout                  = 5000
nstvout                  = 5000
nstlog                   = 250
nstenergy                = 500
nstxtcout                = 250
xtc_grps                 = Protein  SOL  Na+  Cl-
energygrps               = Protein  SOL  Na+  Cl-
nstlist                  = 10
ns_type                  = grid
rlist                    = 1.0
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
optimize_fft             = yes
```

```
rcoulomb                 = 1.0
rvdw                     = 1.0
pbc                      = xyz
tcoupl                   = berendsen
tc-grps                  = Protein  SOL  Na+  Cl-
tau_t                    = 0.1 0.1 0.1 0.1
ref_t                    = 310 310 310 310
annealing                = single single single single
annealing_npoints        = 2 2 2 2
annealing_time           = 0 25     0 25   0 25   0 25
annealing_temp           = 0 310    0 310  0 310  0 310
Pcoupl                   = berendsen
pcoupltype               = anisotropic
tau_p                = 1.0 1.0 1.0 1.0 1.0 1.0
compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
E_z                      = 1 -0.05 1
gen_vel                  = no
gen_temp                 = 310
gen_seed                 = 173529
constraints              = all-bonds
constraint_algorithm     = lincs
unconstrained_start      = no
```

MD Run 2: mm_nachr_md2.mdp, wah_nachr_md2.mdp

```
title                    = wah_nachr_md2
cpp                      = /lib/cpp
define                   = -dBackbone_1000
integrator               = md
dt                            = 0.002
tinit                    = 50.0
nsteps                   = 25000
nstxout                  = 5000
nstvout                  = 5000
nstlog                   = 250
nstenergy                = 500
nstxtcout                = 250
xtc_grps                 = Protein  SOL  Na+  Cl-
energygrps               = Protein  SOL  Na+  Cl-
nstlist                  = 10
ns_type                  = grid
rlist                    = 1.0
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
optimize_fft             = yes
rcoulomb                 = 1.0
rvdw                     = 1.0
pbc                      = xyz
tcoupl                   = berendsen
tc-grps                  = Protein  SOL  Na+  Cl-
tau_t                    = 0.1 0.1 0.1 0.1
ref_t                    = 310 310 310 310
Pcoupl                   = berendsen
pcoupltype               = anisotropic
tau_p                = 1.0 1.0 1.0 1.0 1.0 1.0
compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
E_z                      = 1 -0.05 1
```

```
            gen_vel                  = no
            gen_temp                 = 310
            gen_seed                 = 173529
            constraints              = all-bonds
            constraint_algorithm     = lincs
            unconstrained_start      = no
```

MD Run 3: mm_nachr_md3.mdp, wah_nachr_md3.md

```
            title                    = wah_nachr_md3
            cpp                      = /lib/cpp
            define                   = -dBackbone_500
            integrator               = md
            dt                           = 0.002
            tinit                    = 100.0
            nsteps                   = 25000
            nstxout                  = 5000
            nstvout                  = 5000
            nstlog                   = 250
            nstenergy                = 500
            nstxtcout                = 250
            xtc_grps                 = Protein  SOL  Na+  Cl-
            energygrps               = Protein  SOL  Na+  Cl-
            nstlist                  = 10
            ns_type                  = grid
            rlist                    = 1.0
            coulombtype              = PME
            fourierspacing           = 0.10
            pme_order                = 4
            ewald_rtol               = 1e-5
            optimize_fft             = yes
            rcoulomb                 = 1.0
            rvdw                     = 1.0
            pbc                      = xyz
            tcoupl                   = berendsen
            tc-grps                  = Protein  SOL  Na+  Cl-
            tau_t                    = 0.1 0.1 0.1 0.1
            ref_t                    = 310 310 310 310
            Pcoupl                   = berendsen
            pcoupltype               = anisotropic
            tau_p                = 1.0 1.0 1.0 1.0 1.0 1.0
            compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
            ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
            E_z                      = 1 -0.05 1
            gen_vel                  = no
            gen_temp                 = 310
            gen_seed                 = 173529
            constraints              = all-bonds
            constraint_algorithm     = lincs
            unconstrained_start      = no
```

MD Run 4: mm_nachr_md4.mdp, wah_nachr_md4.mdp

```
            title                    = wah_nachr_md4
            cpp                      = /lib/cpp
            define                   =
            integrator               = md
            dt                      = 0.002
            tinit                    = 150.0
            nsteps                   = 425000
            nstxout                  = 5000
            nstvout                  = 5000
            nstlog                   = 250
            nstenergy                = 500
            nstxtcout                = 250
            xtc_grps                 = Protein  SOL  Na+  Cl-
            energygrps               = Protein  SOL  Na+  Cl-
            nstlist                  = 10
            ns_type                  = grid
            rlist                    = 1.0
            coulombtype              = PME
            fourierspacing           = 0.10
            pme_order                = 4
            ewald_rtol               = 1e-5
            optimize_fft             = yes
            rcoulomb                 = 1.0
            rvdw                     = 1.0
            pbc                      = xyz
            tcoupl                   = berendsen
            tc-grps                  = Protein  SOL  Na+  Cl-
            tau_t                    = 0.1 0.1 0.1 0.1
            ref_t                    = 310 310 310 310
            Pcoupl                   = berendsen
            pcoupltype               = anisotropic
            tau_p                   = 1.0 1.0 1.0 1.0 1.0 1.0
            compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
            ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
            E_z                      = 1 -0.05 1
            gen_vel                  = no
            gen_temp                 = 310
            gen_seed                 = 173529
            constraints              = all-bonds
            constraint_algorithm     = lincs
            unconstrained_start      = no
```

MD Run 5: mm_nachr_md5.mdp, wah_nachr_md5.mdp

```
title                    = wah_nachr_md5
cpp                      = /lib/cpp
define                   =
integrator               = md
dt                      = 0.002
tinit                    = 1850.0
nsteps                   = 425000
nstxout                  = 5000
nstvout                  = 5000
nstlog                   = 250
nstenergy                = 500
nstxtcout                = 250
xtc_grps                 = Protein  SOL  Na+  Cl-
energygrps               = Protein  SOL  Na+  Cl-
nstlist                  = 10
ns_type                  = grid
rlist                    = 1.0
coulombtype              = PME
```

```
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
optimize_fft             = yes
rcoulomb                 = 1.0
rvdw                     = 1.0
pbc                      = xyz
tcoupl                   = berendsen
tc-grps                  = Protein  SOL  Na+  Cl-
tau_t                    = 0.1 0.1 0.1 0.1
ref_t                    = 310 310 310 310
Pcoupl                   = berendsen
pcoupltype               = anisotropic
tau_p                   = 1.0 1.0 1.0 1.0 1.0 1.0
compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
E_z                      = 1 -0.05 1
gen_vel                  = no
gen_temp                 = 310
gen_seed                 = 173529
constraints              = all-bonds
constraint_algorithm     = lincs
unconstrained_start      = no
```

MD Run 6: mm_nachr_md6.mdp, wah_nachr_md6.mdp

```
title                    = wah_nachr_md6
cpp                      = /lib/cpp
define                   =
integrator               = md
dt                      = 0.002
tinit                    = 1850.0
nsteps                   = 425000
nstxout                  = 5000
nstvout                  = 5000
nstlog                   = 250
nstenergy                = 500
nstxtcout                = 250
xtc_grps                 = Protein  SOL  Na+  Cl-
energygrps               = Protein  SOL  Na+  Cl-
nstlist                  = 10
ns_type                  = grid
rlist                    = 1.0
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
optimize_fft             = yes
rcoulomb                 = 1.0
rvdw                     = 1.0
pbc                      = xyz
tcoupl                   = berendsen
tc-grps                  = Protein  SOL  Na+  Cl-
tau_t                    = 0.1 0.1 0.1 0.1
ref_t                    = 310 310 310 310
Pcoupl                   = berendsen
pcoupltype               = anisotropic
tau_p                   = 1.0 1.0 1.0 1.0 1.0 1.0
compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
E_z                      = 1 -0.05 1
gen_vel                  = no
```

```
gen_temp                 = 310
gen_seed                 = 173529
constraints              = all-bonds
constraint_algorithm     = lincs
unconstrained_start      = no
```

MD Run 7: mm_nachr_md7.mdp, wah_nachr_md7.mdp

```
title                    = wah_nachr_md7
cpp                      = /lib/cpp
define                   =
integrator               = md
dt                      = 0.002
tinit                    = 2700.0
nsteps                   = 425000
nstxout                  = 5000
nstvout                  = 5000
nstlog                   = 250
nstenergy                = 500
nstxtcout                = 250
xtc_grps                 = Protein  SOL  Na+  Cl-
energygrps               = Protein  SOL  Na+  Cl-
nstlist                  = 10
ns_type                  = grid
rlist                    = 1.0
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
optimize_fft             = yes
rcoulomb                 = 1.0
rvdw                     = 1.0
pbc                      = xyz
tcoupl                   = berendsen
tc-grps                  = Protein  SOL  Na+  Cl-
tau_t                    = 0.1 0.1 0.1 0.1
ref_t                    = 310 310 310 310
Pcoupl                   = berendsen
pcoupltype               = anisotropic
tau_p                   = 1.0 1.0 1.0 1.0 1.0 1.0
compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
E_z                      = 1 -0.05 1
gen_vel                  = no
gen_temp                 = 310
gen_seed                 = 173529
constraints              = all-bonds
constraint_algorithm     = lincs
unconstrained_start      = no
```

MD Run 8: mm_nachr_md8.mdp, wah_nachr_md8.mdp

```
title                    = wah_nachr_md8
cpp                      = /lib/cpp
define                   =
integrator               = md
```

```
dt                       = 0.002
tinit                    = 3550.0
nsteps                   = 425000
nstxout                  = 5000
nstvout                  = 5000
nstlog                   = 250
nstenergy                = 500
nstxtcout                = 250
xtc_grps                 = Protein  SOL  Na+  Cl-
energygrps               = Protein  SOL  Na+  Cl-
nstlist                  = 10
ns_type                  = grid
rlist                    = 1.0
coulombtype              = PME
fourierspacing           = 0.10
pme_order                = 4
ewald_rtol               = 1e-5
optimize_fft             = yes
rcoulomb                 = 1.0
rvdw                     = 1.0
pbc                      = xyz
tcoupl                   = berendsen
tc-grps                  = Protein  SOL  Na+  Cl-
tau_t                    = 0.1 0.1 0.1 0.1
ref_t                    = 310 310 310 310
Pcoupl                   = berendsen
pcoupltype               = anisotropic
tau_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
E_z                      = 1 -0.05 1
gen_vel                  = no
gen_temp                 = 310
gen_seed                 = 173529
constraints              = all-bonds
constraint_algorithm     = lincs
unconstrained_start      = no
```

MD Run 9: mm_nachr_md9.mdp, wah_nachr_md9.mdp

```
title                    = wah_nachr_md9
cpp                      = /lib/cpp
define                   =
integrator               = md
dt                       = 0.002
tinit                    = 4400.0
nsteps                   = 425000
nstxout                  = 5000
nstvout                  = 5000
nstlog                   = 250
nstenergy                = 500
nstxtcout                = 250
xtc_grps                 = Protein  SOL  Na+  Cl-
energygrps               = Protein  SOL  Na+  Cl-
nstlist                  = 10
ns_type                  = grid
rlist                    = 1.0
coulombtype              = PME
fourierspacing           = 0.10
```

```
           pme_order                = 4
           ewald_rtol               = 1e-5
           optimize_fft             = yes
           rcoulomb                 = 1.0
           rvdw                     = 1.0
           pbc                      = xyz
           tcoupl                   = berendsen
           tc-grps                  = Protein  SOL  Na+  Cl-
           tau_t                    = 0.1 0.1 0.1 0.1
           ref_t                    = 310 310 310 310
           Pcoupl                   = berendsen
           pcoupltype               = anisotropic
           tau_p                   = 1.0 1.0 1.0 1.0 1.0 1.0
           compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
           ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
           E_z                      = 1 -0.05 1
           gen_vel                  = no
           gen_temp                 = 310
           gen_seed                 = 173529
           constraints              = all-bonds
           constraint_algorithm     = lincs
           unconstrained_start      = no
```

MD Run 10: mm_nachr_md10.mdp, wah_nachr_md10.mdp

```
           title                    = wah_nachr_md10
           cpp                      = /lib/cpp
           define                   =
           integrator               = md
           dt                      = 0.002
           tinit                    = 5250.0
           nsteps                   = 425000
           nstxout                  = 5000
           nstvout                  = 5000
           nstlog                   = 250
           nstenergy                = 500
           nstxtcout                = 250
           xtc_grps                 = Protein  SOL  Na+  Cl-
           energygrps               = Protein  SOL  Na+  Cl-
           nstlist                  = 10
           ns_type                  = grid
           rlist                    = 1.0
           coulombtype              = PME
           fourierspacing           = 0.10
           pme_order                = 4
           ewald_rtol               = 1e-5
           optimize_fft             = yes
           rcoulomb                 = 1.0
           rvdw                     = 1.0
           pbc                      = xyz
           tcoupl                   = berendsen
           tc-grps                  = Protein  SOL  Na+  Cl-
           tau_t                    = 0.1 0.1 0.1 0.1
           ref_t                    = 310 310 310 310
           Pcoupl                   = berendsen
           pcoupltype               = anisotropic
           tau_p                   = 1.0 1.0 1.0 1.0 1.0 1.0
           compressibility          = 4.5e-5 4.5e-5 4.5e-5 0 0 0
           ref_p                    = 1.0 1.0 1.0 1.0 1.0 1.0
```

```
E_z                         = 1 -0.05 1
gen_vel                     = no
gen_temp                    = 310
gen_seed                    = 173529
constraints                 = all-bonds
constraint_algorithm        = lincs
unconstrained_start         = no
```

MD Run 11: mm_nachr_md11.mdp, wah_nachr_md11.mdp

```
title                       = wah_nachr_md11
cpp                         = /lib/cpp
define                      =
integrator                  = md
dt                         = 0.002
tinit                       = 6100.0
nsteps                      = 425000
nstxout                     = 5000
nstvout                     = 5000
nstlog                      = 250
nstenergy                   = 500
nstxtcout                   = 250
xtc_grps                    = Protein  SOL  Na+  Cl-
energygrps                  = Protein  SOL  Na+  Cl-
nstlist                     = 10
ns_type                     = grid
rlist                       = 1.0
coulombtype                 = PME
fourierspacing              = 0.10
pme_order                   = 4
ewald_rtol                  = 1e-5
optimize_fft                = yes
rcoulomb                    = 1.0
rvdw                        = 1.0
pbc                         = xyz
tcoupl                      = berendsen
tc-grps                     = Protein  SOL  Na+  Cl-
tau_t                       = 0.1 0.1 0.1 0.1
ref_t                       = 310 310 310 310
Pcoupl                      = berendsen
pcoupltype                  = anisotropic
tau_p                      = 1.0 1.0 1.0 1.0 1.0 1.0
compressibility             = 4.5e-5 4.5e-5 4.5e-5 0 0 0
ref_p                       = 1.0 1.0 1.0 1.0 1.0 1.0
E_z                         = 1 -0.05 1
gen_vel                     = no
gen_temp                    = 310
gen_seed                    = 173529
constraints                 = all-bonds
constraint_algorithm        = lincs
unconstrained_start         = no
```