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Interaction of anesthetics with open and closed conformations of a potassium channel studied via molecular dynamics and normal mode analysis

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ABSTRACT

A variety of experiments suggest that membrane proteins are important targets of anesthetic molecules, and that ion channels interact differently with anesthetics in their open and closed conformations. The availability of an open and a closed structural model for the KirBac1.1 potassium channel, has made it possible to perform a comparative analysis of the interactions of anesthetics with the same channel in its open and closed states. To this end, all-atom molecular dynamics simulations supplemented by normal mode analysis have been employed to probe the interactions of the inhalational anesthetic halothane with both an open and closed conformer of KirBac1.1 embedded in a lipid bilayer. Normal mode analysis on the closed and open channel, in the presence and absence of halothane, reveals that the anesthetic modulates the global as well as the local dynamics of both conformations differently. In the case of the open channel the observed reduction of flexibility of residues in the inner helices, suggests a functional modification action of anesthetics on ion channels. In this context, preferential quenching of the aromatic residue motion and modulation of global dynamics by halothane may be seen as steps towards 'potentiating' or favoring open state conformations. The present molecular dynamics simulations provide the first insights into possible specific interactions between anesthetic molecules and ion channels in different conformations.

INTRODUCTION

Despite the widespread clinical use of anesthetics since the 19th century, a clear understanding of the mechanism of anesthetic action has yet to emerge. The main contenders regarding the site of action for anesthetics are lipids and membrane proteins (MP) underlying the non-specific and specific theories, respectively. Specific interactions of anesthetic molecules with the MPs have been studied since 1952 (1) and experiments have shown that neurotransmitters like $GABA_A$ and glycine receptors are sensitive to clinically relevant concentrations of inhaled anesthetics (2). Photo labeling experiments have revealed possible sites of halothane binding to nicotinic acetylcholine receptors (3). The demonstration of specific action of anesthetics on ion channels through several experiments is a departure from the traditional view of a non-specific mechanism of anesthetic action through their interactions with lipid bilayers. The nature and mechanism of the specific action of anesthetics on ion channels has been discussed extensively in several recent reviews (4-7).

On the computational side, molecular dynamics (MD) simulations have been used to understand the interactions of inhalational anesthetics molecules such as halothane with both model lipid bilayers and ion channels. Simulations of model lipid bilayers demonstrated the preferential distribution of halothane molecules close to the lipid-water interface in agreement with experiments and effects on hydrophobic chain ordering of the lipids (8-10). Other MD simulations of interactions of anesthetic molecules with ion channels include studies of binding of an inhalational anesthetic, halothane, to soluble native and synthetic four-helix bundles (11, 12), to gramicidin in a DMPC membrane (13), to ketosteroid isomerase (14) and to subunits of nicotinic acetylcholine receptors (15).

Ion channels exist in different conformations *viz.,* open, closed resting, inactive. A natural question is the following: do anesthetic molecules interact with these distinct conformations of the same ion channel differently? Addressing the effects of anesthetics on different conformations is difficult experimentally because ion channels remain in an 'open' state for much shorter time than in the 'closed' state. Unfortunately, limited availability of high-resolution ion channel structures, which have been implicated in anesthetic mechanism, has impeded potentially insightful MD simulation studies.

A family of K^+ channels, tandem pore domains, has been shown to be activated by volatile anesthetics (16) and studies on G-protein-gated-inwardly rectifying K (GIRK) channels have shown that these inward rectifiers contain an intrapore binding site for local anesthetics (17). It has been predicted that anesthetics may prolong open-state components. Also, inhalational anesthetics were shown to potentiate open states in ion channels like nicotinic acetylcholine receptor (18). Potassium (K^+) channels are found in a wide range of cells and tissues and play an important role in controlling the resting potential of the membrane through channel opening and closing by selectively transporting K^+ ions across membranes. The KirBac1.1 channel belongs to the inward rectifier family of K^+ channels. The 3D crystal structure of this channel in a closed state at a resolution of 3.65 Å was first reported in 2003 (19). KirBac1.1 has a pore-forming tetrameric transmembrane domain (four monomers are named U1 U2 U3 U4

respectively) similar to that of the prototypical K^+ channel, KcsA. Recently, an open state structure of KirBac1.1 was modeled starting from the closed state and with further refinement using projection maps obtained from electron microscopy experiments (20). The resulting open state structural model was validated through extensive MD simulations (21). We performed additional MD simulations on both the open and closed states of the KirBac1.1 channel in a DOPC lipid membrane environment and showed that the aromatic residues through localization play an important role in directing and stabilizing structural changes within the transmembrane region of this integral membrane protein (22).

The limited time scales of MD simulations (typically a few 10's of nanoseconds) may not capture the global conformational motions of proteins, which play an important role in the function of the protein. Ligands, such as anesthetics upon binding to ion channels or proteins can affect the function by altering their global motions. Structure-based coarse-grained approaches such as normal mode analysis (NMA) have been used to understand effects of ligands on protein dynamics (23), functional motions responsible for catalytic reactions (24-26) and to fit high resolution crystal structures into low-resolution electron density maps (27). In the coarse-grained elastic network models used in NMA, the residues are represented as nodes in the network with complex force-field interactions between them replaced by harmonic potentials with a cut-off (28-30). The collective modes representing global (delocalized) changes have been shown to be adequately represented by few low frequency modes (31), which are well captured by such coarse-grain models. In the current work, an elastic network model based on Gaussian-distributed fluctuations (referred to as the Gaussian network model or GNM) is used to understand the effects of halothane molecules on the functional motions of both closed and open conformations of the KirBac1.1 ion channel. For comparative analysis, we have performed the NMA on crystal structure of closed and model of open conformations as well as equilibrated MD structures of liganded and unliganded ion channels as inputs. KirBac1.1 provides us with a unique opportunity to compare the effects of inhaled anesthetics on two different conformations within the same family. In this study, we focus on how inhaled anesthetics interact with certain key aromatic residues, as well as influence both local and global protein dynamics in the closed and open conformations of this channel.

METHODS

Description of the systems

The starting configurations before addition of halothane molecules were taken from our previous MD simulations (20 ns for each conformation) and details of setting up closed and open systems can be found in the supplementary information of that work (22). Since in this study we are interested in understanding the effects of halothane molecules on the transmembrane domain of the KirBac channel, we retained the slide helices and the transmembrane (TM) portions and did not include the intracellular domain. In the present MD simulations, 80 halothane molecules were added to the water phase. This concentration of the halothane molecules, which is much higher than clinical concentration, is needed to improve statistics concerning the behavior of the anesthetics. The halothane molecules were initially inserted near the head groups of both the inner

and outer membrane leaflets. This was done because spontaneous translocation of the anesthetic molecules across the lipid membrane is not likely within the MD simulation time (~15ns). Each system was then subjected to additional energy minimization runs using the conjugate gradient method, followed by MD equilibration runs.

Molecular dynamics simulations

MD simulations were performed using the NAMD2 (32) software package. The Nosé-Hoover method with Langevin dynamics and Langevin piston were applied to maintain a pressure of 1 bar and temperature of 305 K. A multiple time step (33, 34) was used and all the hydrogen atoms were constrained using the RATTLE algorithm to allow a time step of 1.5 fs. The CHARMM 22 (35) and CHARMM 27 (36) force-fields were used for the peptide bundles and lipids, respectively and water was modeled by TIP3P (37). Parameters developed by Scharf and Laasonen (38) were employed for halothane molecules. A cut-off distance of 12 Å and a pair-list distance of 15 Å were used to compute all non-bonded interactions and periodic boundary conditions were imposed. The full electrostatics interactions were computed with the Particle Mesh Ewald (PME) method with a tolerance of 10^{-6} and updated every two time steps.

Normal mode analysis (NMA)

The elastic network models in NMA are based on random polymer network models (39). As described in the Introduction, in this approach, protein residues are coarse-grained and each residue (represented by C_α) becomes a node in this three dimensional network. The underlying assumption in elastic network models is that the protein under consideration represents the 'native state' and the global motions can be derived from superposition of various normal modes assuming the harmonic approximation. In the GNM, used in this study, the nodes (hence the residues) are subjected to Gaussian fluctuations (40). In detail, a connectivity matrix (Kirchoff) matrix ($\mathbf{\Gamma}$) is constructed based on a cut-off distance (r_C) as

$$\Gamma_{ij} = \begin{cases} -1 & \text{if } i \neq j,\ r_{ij} \leq r_c \\ 0 & \text{if } i \neq j,\ r_{ij} > r_c \\ \sum_{i \neq j} \Gamma_{ij} & \text{if } i = j \end{cases}$$

By diagonalization of the Kirchoff matrix, a set of eigen values and eigen modes are obtained. The root mean squared fluctuations (RMSF) of the residues of the protein are obtained from the inverse of the Kirchoff matrix as

$$\langle \Delta r_i^2 \rangle = \frac{3k_B T}{\gamma} [\Gamma^{-1}]_{ii}$$

Here, γ is parameter of the Hookean potential and serves as a scaling factor for the comparison of fluctuations from the simulation and experiments (if B-factors are available) (40), k_B is the Boltzmann constant and T is the temperature in the elastic network. The RMSF values of residues are indicative of the mobility of those residues, and the terms RMSF and mobility will be used interchangeably in the text. The above

equation indicates that the connectivity of a node (or a residue) is inversely proportional to the flexibility of the node. In this regard, the RMSF values depend on the value of r_C. A cut-off value less than 10 Å yielded more than one zero eigen values for the systems with halothane rendering the results unreliable. Convergence studies on the optimal value of r_C yielded 15 Å for all the systems studied in this work. It is to be noted that the eigen modes from the Kirchoff matrix do not have information about the direction of motion. A Hessian matrix, whose elements are second derivatives of the spring potential used to connect the nodes of the GN, needs to be constructed for the directional fluctuations of the individual residues, which is implemented in anisotropic network model (ANM) (41). Both GNM and ANM have online implementations and can be found at (ignm.ccbb.pitt.edu). Halothane molecules were included in the NMA computations as described in the recent work of Szarecka *et al* (23). More details on the inclusion of halothane molecules in the GNM calculations are described in the results section.

RESULTS AND DISCUSSION

Characterization of membrane-protein system

Within ~15 ns, most of the halothane molecules initially placed in the solution phase spontaneously partitioned into the lipid-protein system. In Fig. 1, the partitioning of halothane molecules for both the closed and open channel systems is shown at t = 0 and t = 15 ns, respectively. The partitioning of halothane molecules is asymmetric with respect to the membrane normal (z-axis) in both systems. However, we observed a higher preference for the halothane molecules to be concentrated near the entry of the channel pore at the intracellular region of the open conformation, which is not the case in the closed conformation. About 75% of halothane molecules (60 out of 80) are close to the head group region in inner lipid leaflet in the open conformation as opposed to somewhat equal distribution of the halothane molecules in both side lipid leaflets in the closed conformation.

In previous bilayer-halothane computational studies, the most favored locations of halothane molecules is just below the polar head groups in a complex interfacial region of hydrophilic head groups and hydrophobic tail groups reflecting the amphiphilic nature of the halothane molecules(8, 9, 15, 42, 43). The spontaneous partitioning of halothane molecules into bilayers in times accessible to computer simulations was shown, using steered molecular dynamics calculations, to be due to the absence of significantly high energy barriers against entry into the bilayer using steered molecular dynamics calculations (15). In the current simulations also, as described in the previous section, halothane molecules preferentially target the locations below the head group region. The presence of anesthetic molecules near the interface of the membrane protein and lipid bilayer, thus disrupting the protein-lipid contacts has been observed before (13). In the current simulations anesthetics molecules also partitioned such that they are located near the protein-lipid interface. The number of halothane molecules near the protein-head group interface in the simulations of the closed conformation was higher than those of the open conformation.

We have also investigated the distribution of halothane molecules around the protein and in both conformations; halothane molecules are mostly concentrated close to aromatic residues. Density profiles of halothane molecules projected along the bilayer normal (z-direction) and averaged over the last 6 ns of the simulation are shown in Fig. 2. The distribution of aromatic residues significantly changes in the presence of halothane molecules in both the open and closed conformations. Importantly, the width of the TRP residue distribution peak (near the inner leaflet) is reduced and is sharper in the presence of halothane molecules suggesting increase in the localization of these residues.

Fluorescence experiments (44) have suggested that halothane molecules quench the motion of TRP residues reducing the fluctuations in the motion of the indole ring. Similar modulation of protein motion in the presence of halothane molecules was observed in previous MD simulations (11, 13). The halothane molecule distribution (magnified by 5 times for clarity) is also shown in Fig 2. From the distribution we can see that near the entry of the channel pore at the intracellular side of the closed conformation, the anesthetic molecules are occluded (~-20 Å), whereas in the open conformation, the distribution peaks near the intracellular pore entry.

Previous atomistic MD simulations showed the distribution of halothane molecules at low anesthetic concentration to be essentially bimodal in nature (8, 9, 43), with halothane molecules preferentially occupying sites close to the interfacial head-group water region. However, in recently performed coarse-grain (CG) simulations (10), it was seen that this bimodal distribution of halothane molecules is not valid if the concentration of the anesthetic molecules is high. In the latter study, it was observed that as the concentration of the CG halothane molecules increased, there was an appreciable presence of halothane molecules at the center of the hydrophobic hydrocarbon region. In the present simulations also, we observe that halothane molecules penetrate the lipid bilayer and have an appreciable presence at the bilayer center, as can be seen from Fig.2.

In the open conformation, the halothane molecules were observed to be concentrated at the entry of the channel near the intracellular domain, which is not the case for the closed conformation. Figure 3 shows average representations of the open and closed conformations of the pore region both with and without halothane molecules. The entry of the intracellular pore in the closed conformation remains ‘occluded’ to anesthetic molecules throughout the 15ns MD simulation time and on an average six halothane molecules are found near the pore region. The pore of the closed conformation has four PHE residues occluding the pore and more aromatic residues (including TRP and TYR) line the spacing between the inner and outer loops. On the other hand, in the case of the open conformer, on average 25 halothane molecules are found near and in the pore region as the inner loops open up. One of the effects of the presence of the anesthetic molecules on the open conformer is to reduce the flexibility of the inner helices near the intracellular pore entry.

To understand the effects of halothane on the local dynamics of the open and closed conformations, root mean square fluctuation (RMSF) calculations were performed. In Fig.4, the RMSF calculations of open and closed conformations without the slide helices

are shown. The overall shapes of the RMSF profiles in the case of both open and closed conformations in the presence and absence of halothane molecules is somewhat similar. In both cases an increase in the flexibility of the loops, which are close to the lipid-protein interface has been observed. Halothane molecules were found close to these loops and as suggested in previous simulations (13, 14), the increase in flexibility can be related to perturbation of lipid-protein interactions due to presence of anesthetic molecules. We also looked at the effect of halothane on the lipid-protein interaction and it was seen that in the presence of halothane molecules, there is an appreciable decrease in the contacts in case of TRP and TYR aromatic residues in both open and closed conformations, whereas, there is essentially no difference in the contacts in the case of PHE residues (data not shown). It has also been observed that the presence or absence of anesthetics has no appreciable effect on the mobility of the selectivity filter.

Anesthetic modulation of global dynamics

The total length of the MD simulation for each of the conformations is ~ 15ns. Though this time scale may capture atomistic details of anesthetic interactions with different conformations of the ion channel, this time is likely to be too short to capture the effect on global dynamics, if any, of the anesthetic molecules. We calculated the low frequency global modes for the crystal structure as well as the MD equilibrated structure of the closed form of the KirBac1.1 channel. The average of three slowest modes will be henceforth referred to as the global mode. Our results, shown in Fig. 5, agree very well with those obtained in the work of Shrivastava *et al.* (45) on pore opening mechanisms of different potassium channels.; this is perhaps not surprising as the same Gaussian model was used (especially the results for the closed conformation). The residue mobilities in the low frequency global mode of the MD equilibrated structure overlap very well with those of the crystal structure, even though the four-fold degeneracy as exhibited by the highly symmetric crystal structure is somewhat lost in the MD. The selectivity filter and the GLY residues (equivalent to GLY134 in the crystal structure (19)) of inner helices are the minima of the global modes, as observed previously (45, 46). The global minimum at GLY residues suggests that the residue may serve as hinge site for the protein, which was seen in a previous simulation (46) looking at the conformational flexibility of the inner helices of KirBac1.1. We have also plotted the residue mobilities in the global mode for the open model and MD equilibrated structures. The global minimum near the selectivity filter is still preserved in the open conformation. The MD equilibrated structure in the open configuration exhibits more mobility in the N-terminal residues as compared to the model. In a previous work (46), it has been shown that the inner helices (M2 helices) of KirBac channels exhibit dimer-of-dimers type of motion in both octane and a POPC membrane. In the present simulation, the KirBac channels in closed-form exhibit dimer-of-dimers motion both in the absence and presence of halothane molecules (Figure 1S in supplementary material).

As described in the methods section, the halothane molecules are also included in the NMA calculations as single nodes to understand the effect of anesthetics on functional modes and dynamics of KirBac1.1 channels. The starting configurations for the GNM are taken from structures averaged over the last 1 ns of the MD trajectory. In the GNM calculations only halothane molecules near the entry of intracellular pore are included in

both conformations. The mobilities of the closed- and open-conformations in the presence and absence of halothane molecules have been mapped to the structure and are shown in Fig. 6. The color coding used in the figure ranges from red (most mobile) to green (intermediate) and to blue (least mobile). The halothane molecules included in the calculation are also included in the figure. From the figure, it is very clear that the presence of halothane molecules near the intracellular pore entry in open conformation, has a significant effect of quenching the motion of residues as compared to the closed conformation.

The mobilities of the closed conformation in the presence and absence of halothane molecules are not significantly different as can be seen in Fig. 6. Due to the constriction at the entry of the pore near the intracellular side of the closed channel, the number of halothane molecules entering and hence affecting the inner helices (where the gate is located) is much smaller. In contrast, the mobilities of inner helices are significantly affected by the presence of halothane molecules in the case of the open conformation, as shown in Fig. 6. Due to the favorable pore space available in the open conformation, a significant number of halothane molecules are found inside and near the intracellular pore entry. From the crystal structure calculations (19), the KirBac1.1 channel in closed conformation is understood to prevent K^+ ion conduction by occluding the pore entry from the intracellular side using the phenylalanine residues that are the blocking residues near the gate of the channel. The flexibility of the open channel, especially that of the PHE residues on the inner helices, has been implicated in playing a crucial role in channel gating (22). The present normal mode analysis does reveal that the anesthetic molecules affect the open conformation of the KirBac1.1 channel by reducing the mobility of the inner helices. The decrease in flexibility in the region close to the entry of the channel pore may play a role in potentiating the open conformation in the presence of halothane molecules. The root mean square fluctuations of the protein residues in the global mode in the presence and absence of halothane molecules is shown in Fig. 7. The global minima near the selectivity filter (indicated by arrows) remain unaltered in both conformations in the presence of halothane molecules also.

The ANM calculations reveal the direction of motions involved in the slowest normal modes. As in the GNM calculations, only halothane molecules near the intracellular pore of the proteins were included in the ANM calculations. Halothane molecules do not significantly affect the direction of motion of first three global modes of the closed conformation. However, in the open conformation, halothane molecules significantly alter the direction of motion of the third slowest global mode. This mode contains hinge-like symmetric movement of outer and inner helices involving dimer-of-dimer motions (U1–U3 & U2-U4). These symmetric motions may play an important role in gating of the KirBac1.1 channel (19), required for transition between open and closed conformations. Addition of halothane molecules to the system changes these motions from symmetric to anti-symmetric. This change of direction may hamper the gating transition and along with the reduction of flexibility as described earlier may prolong the open state. In the open conformation of the KirBac1.1 ion channel, the cavity near the intracellular pore entry flanked by aromatic residues (hydrophobic environment) and polar head groups and water (polar environment) seem an appropriate location for binding of anesthetics as

observed in the current MD simulation. Once the anesthetics occupy this cavity, they affect the functional modes of the open conformation as described in this section.

Aromatic localization and anesthetics

In our previous simulations (22) we have observed that the aromatic residues, especially PHE, close to the lipid-protein interface may play a role in controlling channel gating through localization. In our current MD simulations we explore the dynamic nature of the aromatic residues in the closed and open conformations with and without halothane molecules. An order parameter, characterizing the motion of the aromatic residues, S_N is defined as the angle between the normal to the bilayer and a vector drawn perpendicular to the aromatic ring. We evaluated the histogram distribution of the order parameter over the last 8ns for both closed and open conformers in the presence and absence of halothane. From the comparative analysis, we find that in the case of the closed conformer, the effect of the presence of halothane molecules on the order parameter, S_N is negligible. In the case of the open conformation, however, for the PHE residues near the pore of the channel, where halothane molecules accumulate in the MD simulation, there is an appreciable quenching of the motion of the aromatic rings. Order parameter results for three such residues as shown in Fig. 8.

The implication of such quenching is important for potentiating the open state. In this particular case of the KirBac channel, we have shown earlier that the orientation of the PHE residues close to the lipid-protein interface is very dynamic and changes drastically over the MD simulation time (~10ns) as compared to the closed conformation (22). The dynamic nature of the PHE orientation is thought to play a role in preventing the channel from being stabilized in the open state. It was suggested earlier (47) that one of the fundamental effects of volatile anesthetics on protein action may be through attenuation of local side-chain dynamics and stabilization of certain folded protein conformations.

CONCLUDING REMARKS

There exists an intimate relationship between protein function and flexibility. Fluorescence anisotropy measurements among other experimental techniques are able to provide insights into the physical effects of anesthetics in proteins. It has been shown that anesthetics may alter protein function by decreasing protein flexibility and stabilizing certain protein conformations (47-49). Simulations of halothane with gramicidin, luciferase (13, 23) have suggested that anesthetic molecules modulate the protein global dynamics. Simulations of halothane interactions with subunits of nicotinic acetyl choline receptor have shown that the anesthetic molecules modulate the dynamics of loops connecting the M2 and M3 subunits, a loop that is implicated in gating mechanism (15, 50) by allosterically transmitting the effects to channel gate. Likewise, in the current MD simulations we see that halothane molecules influence both the local and global dynamics of different conformations but clearly affect the open conformer more. Halothane molecules target the inner helices of the open conformer and decrease the flexibility significantly as revealed by first three slowest global normal modes. A gate in the KirBac1.1 channel is located at the intracellular pore entry and the four phenylalanine residues form the channel gate. The KirBac1.1 is a unique channel in that it has an abundance of PHE residues. The presence of aromatic residues close to the lipid head

groups have been implicated in stable insertion of the channel using favorable (cation-pi) interactions between polar head groups and aromatic rings. We have previously demonstrated, through multiple ns MD simulations, that through the process of aromatic localization structural changes during conformational transitions within the transmembrane region of KirBac are stabilized(22). As these aromatic residues are conserved throughout the Kir family, we proposed that aromatic localization could contribute to the forces driving conformational changes. From the analysis of order parameters measuring the flexibility of the aromatic residues, it was observed that the PHE residues near the interface experience much higher mobility compared to the closed conformation in the absence of any anesthetic molecules. From that study, the entropy associated with the increased flexibility of the aromatic residues in the open conformer was implicated in preventing the stabilization of the open conformer. With the addition of halothane molecules, we see that the mobility of the inner helices and PHE residues near the gate is significantly decreased. A unitary explanation of anesthetic action on proteins by Tang *et al* (13) suggests that functionally significant changes caused by anesthetics are a result of protein's intrinsic susceptibility to the presence of such modulators as is also seen here.

Cavities inside proteins, especially those embedded in membranes, with appropriate mixture of hydrophobic and polar environment have been implicated as possible locations for anesthetic binding (49). Binding of hydrophobic molecules in cavities of proteins has been shown to reduce the protein motion (51). The binding of anesthetics in the cavities of a protein is expected to play a role in preferentially stabilizing those conformers, which are otherwise poorly populated (49). Membrane proteins spend more time in closed conformations than in open ones. The open conformations, on the other hand possess cavities large enough to bind small hydrophobic molecules such as anesthetics. This scenario is very close to the conformational ensemble concept described above. The current study suggests that the anesthetic molecules, by preferentially occupying the available (and favorable) cavity in the open conformation, reduce the protein motion in that vicinity and thereby affect the residues implicated in the gating of the channel. This in turn may eventually potentiate the open conformer, consistent with the attractive cavity hypothesis. Simulations with non-immobilizers and other anesthetic molecules on different conformations of membrane proteins may shed further light on the underlying principles of anesthetic action.

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References

1. Larrabee, M. G., and J. M. Postenack. 1952. Selective action of anesthetics on synapses and axons in mammalian symathetic ganglia. *J Neurophysiol* 15:91-114.
2. Mihic, S. J., Q. Ye, M. J. Wick, V. V. Koltchine, M. A. Krasowski, S. E. Finn, M. P. Mascia, C. F. Valenzuela, K. K. Hanson, E. P. Greenblatt, R. A. Harris, and N. L. Harrison. 1997. Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* 389:385-389.
3. Chiara, D. C., L. J. Dangott, R. G. Eckenhoff, and J. B. Cohen. 2003. Identification of nicotinic acetylcholine receptor amino acids photolabeled by the volatile anesthetic halothane. *Biochemistry* 42:13457-13467.
4. Yamakura, T., E. Bertaccini, J. R. Trudell, and R. A. Harris. 2001. Anesthetics and ion channels: Molecular models and sites of action. *Annu. Rev. Pharmacol. Toxicol.* 41:23-51.
5. Dilger, J. P. 2002. The effects ofgeneral anetshetics on ligand-gates ion channels. *Br. J. Anaesth.* 89:41-51.
6. Campagna, J. A., K. W. Miller, and S. A. Forman. 2003. Mechanisms of actions of inhaled anesthetics. *N. Engl. J. Med.* 348:2110-2124.
7. Miller, K. W. 2002. The nature of sites of general anesthetic action. *Br. J. Anaesth.* 89:17-31.
8. Tu, K. C., M. Tarek, M. L. Klein, and D. Scharf. 1998. Effects of anesthetics on the structure of a phospholipid bilayer: Molecular dynamics investigation of halothane in the hydrated liquid crystal phase of dipalmitoylphosphatidylcholine. *Biophys. J.* 75:2123-2134.
9. Koubi, L., M. Tarek, M. L. Klein, and D. Scharf. 2000. Distribution of halothane in a dipalmitoylphosphatidylcholine bilayer from molecular dynamics calculations. *Biophys. J.* 78:800-811.
10. Pickholz, M., L. Saiz, and M. L. Klein. 2005. Concentration effects of volatile anesthetics on the properties of model membranes: A coarse-grain approach. *Biophys. J.* 88:1524-1534.
11. Davies, L. A., Q. F. Zhong, M. L. Klein, and D. Scharf. 2000. Molecular dynamics simulation of four-alpha-helix bundles that bind the anesthetic halothane. *Febs Letters* 478:61-66.
12. Davies, L. A., M. L. Klein, and D. Scharf. 1999. Molecular dynamics simulation of a synthetic four-alpha-helix bundle that binds the anesthetic halothane. *Febs Letters* 455:332-338.
13. Tang, P., and Y. Xu. 2002. Large-scale molecular dynamics simulations of general anesthetic effects on the ion channel in the fully hydrated membrane: the implication of molecular mechanisms of general anesthesia. *Proc. Natl. Acad. Sci. U. S. A* 99:16035-16040.
14. Yonkunas, M. J., Y. Xu, and P. Tang. 2005. Anesthetic interaction with ketosteroid isomerase: insights from molecular dynamics simulations. *Biophys. J.* 89:2350-2356.
15. Vemparala, S., L. Saiz, M. F. Eckenhoff, and M. L. Klein. 2006. Partitioning of anesthetics into a lipid bilayer and their interaction with membrane-bound peptide bundles. *Biophys. J.* 91:2815-2825.

16. Yost, C. S. 2000. Tandem pore domain K channels: an Important site of volatile anesthetic action? *Curr. Drug Targets* 1:207-217.
17. Slesinger, P. A. 2001. Ion selectivity filter regulates local anesthetic inhibition of G-protein-gated inwardly rectifying K^+ channels. *Biophys. J.* 80:701-718.
18. Forman, S. A., K. W. Miller, and G. Yellen. 1995. A discrete site for general anesthetics on a polysynaptic receptor. *Mol. Pharmacol.* 48:574-581.
19. Kuo, A., J. M. Gulbis, J. F. Antcliff, T. Rahman, E. D. Lowe, J. Zimmer, J. Cuthbertson, F. M. Ashcroft, T. Ezaki, and D. A. Doyle. 2003. Crystal structure of the potassium channel KirBac1.1 in the closed state. *Science* 300:1922-1926.
20. Kuo, A., C. Domene, L. N. Johnson, D. A. Doyle, and C. Venien-Bryan. 2005. Two different conformational states of the KirBac3.1 potassium channel revealed by electron crystallography. *Structure* 13:1463-1472.
21. Domene, C., D. A. Doyle, and C. Venien-Bryan. 2005. Modeling of an ion channel in its open conformation. *Biophys. J.* 89:L01-L03.
22. Domene, C., S. Vemparala, M. L. Klein, C. Venien-Bryan, and D. A. Doyle. 2006. Role of aromatic localization in the gating process of a potassium channel. *Biophys. J.* 90:L01-L03.
23. Szarecka, A., Y. Xu, and P. Tang. 2007. Dynamics of firefly Luciferase inhibition by general anesthetics: gaussian and anisotropic network analysis. *Biophys. J.* 93:1895-1905.
24. Kurt, N., W. R. P. Scott, C. A. Schiffer, and T. Haliloglu. 2003. Cooperative fluctuations of unliganded and substrate-bound HIV-1 protease: a structure-based analysis on a variety of conformations from crystallography and molecular dynamics simulations. *Proteins: Struct, Funct, Bioinf* 51:409-422.
25. Delarue, M., and Y. H. Sanejouand. 2002. Simplified normal mode analysis of conformational transitions in DNA-dependent polymerases: The elastic network model. *J. Mol. Biol.* 320:1011-1024.
26. Yang, L. W., and I. Bahar. 2005. Coupling between catalytic site and collective dynamics: a requirement for mechanochemical activity of enzymes. *Structure* 13:893-904.
27. Suhre, K., and Y. H. Sanejouand. 2004. On the potential of normal mode analysis for solving difficult molecular replacement problems. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 60:796-799.
28. Tirion, M. M. 1996. Large amplitude elastic motions in proteins from a single-parameter atomic analysis. *Phys Rev Lett* 77:1905-1908.
29. Bahar, I., A. R. Atilgan, and B. Erman. 1997. Direct evaluation of thermal fluctuations in proteins using a single-parameter harmonic potential. *Folding and Design* 2:173-181.
30. Tama, F., and Y. H. Sanejouand. 2002. Conformational change of proteins arising from normal mode calculations. *Protein Eng.* 14:1-6.
31. Isin, B., P. Doruker, and I. Bahar. 2002. Functional motions of influenza virus hemagglutinin: A structure based analytical approach. *Biophys. J.* 82:569-581.
32. Kale, L., R. Skeel, M. Bhandarkar, R. Brunner, A. Gursoy, N. Krawetz, J. Philips, A. Shinozaki, K. Varadarajan, and K. Schulten. 1999. Molecular dynamics programs design-NAMD2: greater scalability for parallel molecular dynamics. *J. Comput. Phys.* 151:283.

33. Martyna, G. J., M. E. Tuckerman, D. J. Tobias, and M. L. Klein. 1996. Explicit Reversible Integrators for Extended Systems. *Mol Phys* 87:1117-1157.
34. Tuckerman, M. E., and G. J. Martyna. 2000. Understanding Modern Molecular Dynamics: Techniques and Applications. *J Phys Chem B* 104:159-178.
35. MacKerell, A. D., D. Bashford, M. Bellott, R. L. Dunbrack, J. D. Evanseck, M. J. Field, S. Fischer, J. Gao, H. Guo, S. Ha, D. Joseph-McCarthy, L. Kuchnir, K. Kuczera, F. T. K. Lau, C. Mattos, S. Michnick, T. Ngo, D. T. Nguyen, B. Prodhom, W. E. Reiher, B. Roux, M. Schlenkrich, J. C. Smith, R. Stote, J. Straub, M. Watanabe, J. Wiorkiewicz-Kuczera, D. Yin, and M. Karplus. 1998. All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J Phys Chem B* 102:3586-3616.
36. Feller, S. E., and A. D. MacKerell. 2000. An improved empirical potential energy function for molecular simulations of phospholipids. *J Phys Chem B* 104:7510-7515.
37. Jorgensen, W. L., J. Chandrasekhar, J. D. Madura, R. W. Impey, and M. L. Klein. 1983. Comparison of Simple Potential Functions for Simulating Liquid Water. *J. Chem. Phys.* 79:926-935.
38. Scharf, D., and K. Laasonen. 1996. Structure, effective pair potential and properties of halothane. *Chem. Phys. Lett.* 258:276-282.
39. Flory, P. J. 1976. Statistical thermodynamics of random networks. *Proceedings of Royal Society of London A* 351:351-380.
40. Haliloglu, T., I. Bahar, and B. Erman. 1997. Gaussian dynamics of folded proteins. *Phys Rev Lett* 79:3090-3093.
41. Atilgan, A. R., S. R. Durell, R. L. Jernigan, M. C. Demirel, O. Keskin, and I. Bahar. 2001. Anisotropy of fluctuation dynamics of proteins with an elastic network model. *Biophys. J.* 80:505-515.
42. Tang, P., B. Yan, and Y. Xu. 1997. Different distribution of fluorinated anesthetics and non-anesthetics in model membranes a 19F NMR study. *Biophys. J.* 72:1676-1682.
43. Koubi, L., L. Saiz, M. Tarek, D. Scharf, and M. L. Klein. 2003. Influence of anesthetic and nonimmobilizer molecules on the physical properties of a polyunsaturated lipid bilayer. *J Phys Chem B* 107:14500-14508.
44. Tsao, D. H. H., J. R. Casa-Finet, A. H. Maki, and J. W. Chase. 1989. Triplet stste properties of tryptophan residues in complexes of mutated Escherichia Coli single-stranded DNA binding proteins with single-stranded polynucleotides. *Biophys. J.* 55:927-936.
45. Shrivastava, I. H., and I. Bahar. 2006. Common mechanism of pore opening shared by five different potassium channels. *Biophys. J.* 90:3929-3940.
46. Grottesi, A., C. Domene, B. Hall, and M. S. P. Sansom. 2005. Conformational dynamics of M2 helics in KirBac channels: helix flexibility in relation to gating via molecular dynamics simulations. *Biochemistry* 44:14586-14594.
47. Johansson, J. S., H. Zou, and J. W. Tanner. 1999. Bound volatile general anesthetics alter both local protein dynamics and global protein stability. *Anesthesiology* 90:235-245.

48. Ueda, I., and A. Suzuki. 1998. Irreversible phase transition of firefly Luciferase: cotrasting effects of volatile anesthetics and myristic acid. *Biochim. Biophys. Acta* 1380:313-319.
49. Eckenhoff, M. F. 2001. Promiscuous ligands and attractive cavities: how do the inhaled anesthetics work? *Molecular Interventions* 1:258-268.
50. Law, R. J., R. H. Henchman, and J. A. McCammon. 2005. A gating mechanism proposed from a simulation of a human 7 nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. U. S. A* 102:6813-6818.
51. Morton, A., and B. W. Matthews. 1995. Specificity of ligand binding in a buried nonpolar cavity of T4 lysozyme: linkage of dynamics and structural plasticity. *Biochemistry* 34:8576-8588.

Figure Legends

Figure 1: Snapshots taken from MD simulations of the closed (upper) and open (lower) conformations of the KirBac1.1 ion channel embedded in a lipid bilayer at t = 0 (left) and t = 15ns (right). The protein backbones are rendered as ribbons with aromatic residues drawn in white and the halothane molecules in stick representation. The lipid headgroups are also shown and indicate the membrane-water interface. The lipid hydrocarbon chains and water are not shown for visual clarity.

Figure 2: The density profiles along the bilayer normal, z, averaged over last 6ns of the MD trajectory for closed and open conformations of KirBac1.1 (z = 0 represents the center of the bilayer). The distribution of halothane molecules is magnified 5 times for clarity. Distributions of different aromatic residues, charged residues and lipid molecules are also shown.

Figure 3: Shown in the upper left and right panels are the average closed and open conformations, respectively of KirBac taken from the MD simulations and viewed looking down the selectivity filter from extracellular side. The protein backbone alpha-helices are rendered as ribbons. Only the key aromatic residues lining the pore are shown. Halothane molecules that are found near the pore are shown in the bottom two panels. In the case of the closed conformation (bottom, left), the pore restricts entry of halothane molecules but in the case of the open conformer (bottom, right), on an average, 25 halothane molecules are present.

Figure 4: Influence of anesthetics on the dynamical behavior of the KirBac ion channel. Shown is a comparison of the computed root mean squared fluctuations (RMSF) of the backbone Cα atoms for the closed (upper) and open (lower) conformations in the presence and absence of halothane molecules, respectively. The subunits in both the conformations are named as U1, U2, U3 and U4.

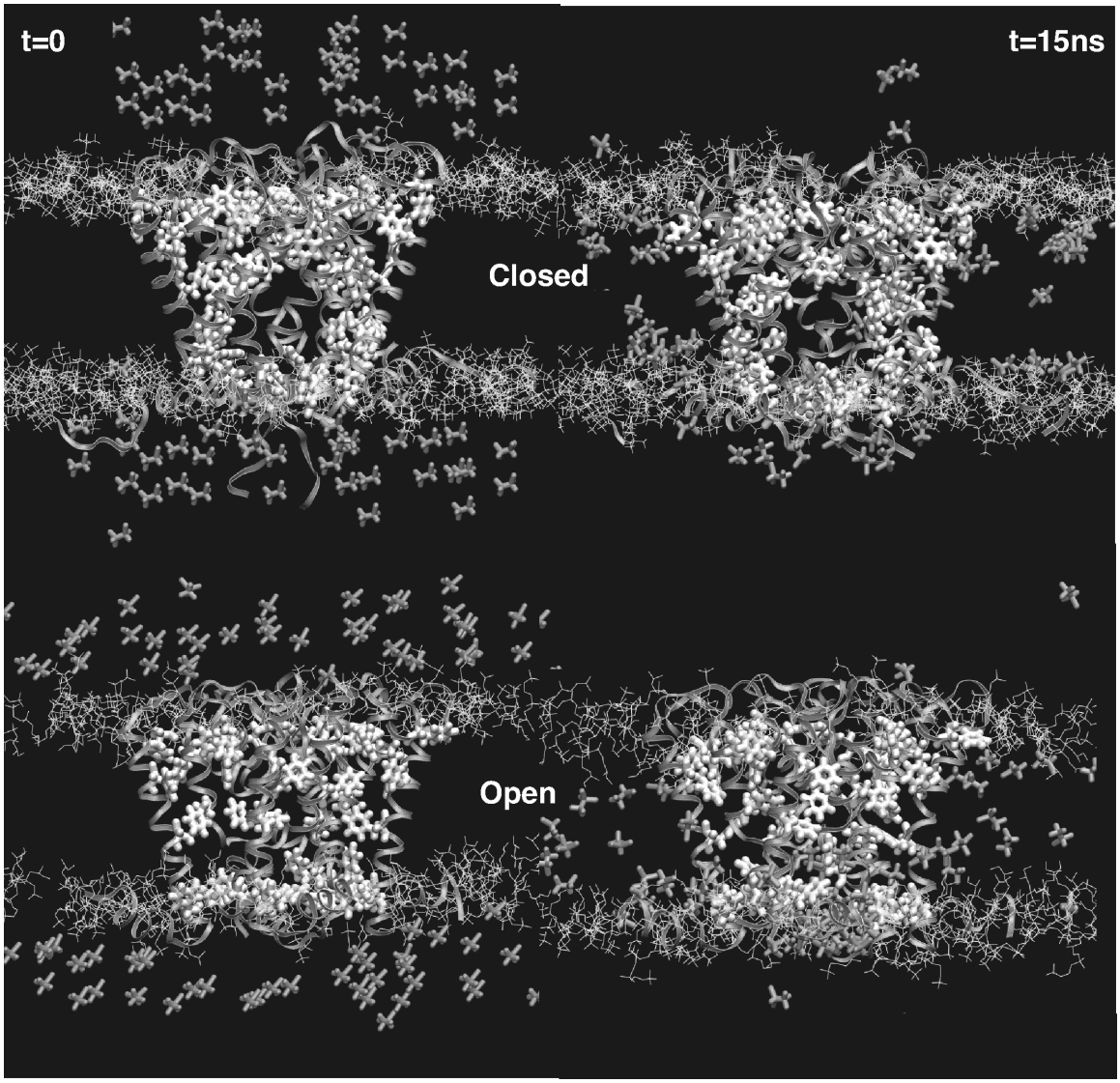
Figure 5: Comparison of mean square fluctuations of residues in the global mode (average of three slowest modes) for closed (top) and open (bottom) conformations between the crystal/model and equilibrated MD structures in the absense of halothane molecules from IGNM calculations are shown. The arrows represent the minimum at the selectivity filter (darker) and at the GLY kink in M2 helix (lighter).

Figure 6: Influence of anesthetics on the dynamical behavior of the KirBac 1.1 ion channel. The mobilities of the residues in the global mode are mapped onto the ribbon representation of the closed and open conformations. The color-coding is from red (most mobile) to blue (least mobile). The halothane molecules represented as single nodes in the GNM calculations are also shown in yellow.

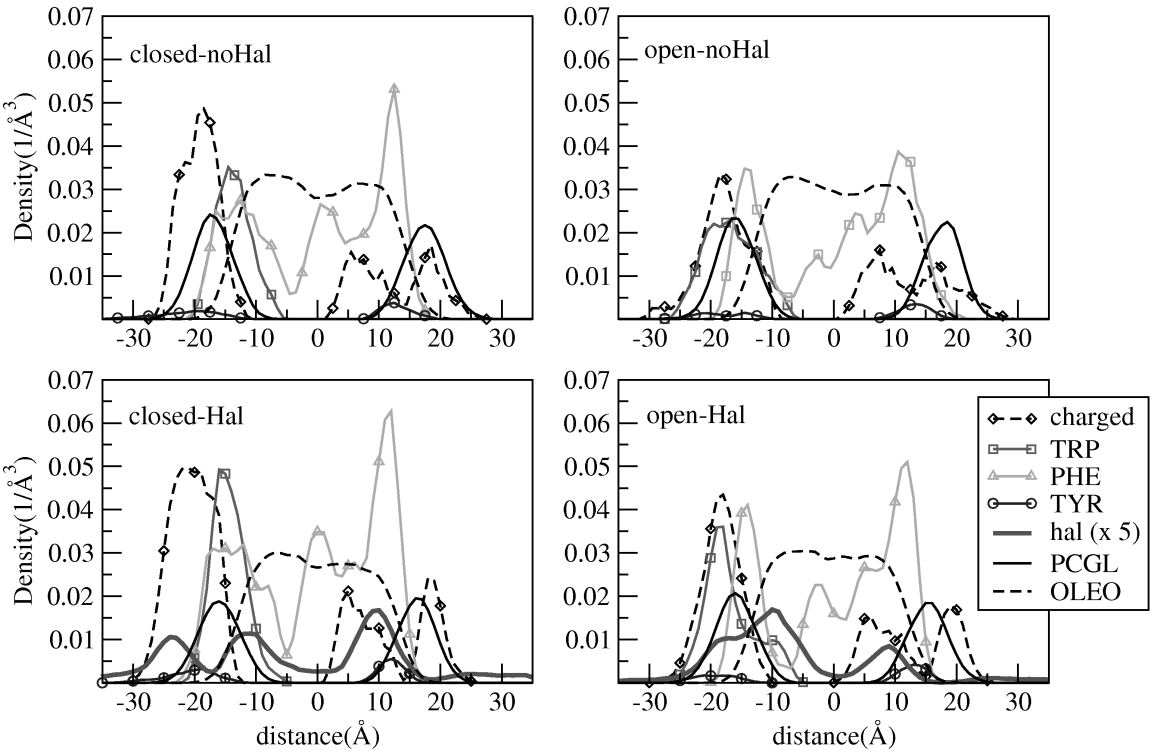
Figure 7: The mean square fluctuations of the residues in the global mode in the presence and absence of halothane molecules is shown. The arrows represent the minimum at the selectivity filter (darker) and at the GLY kink in M2 helix (lighter). Only

halothane molecules near the entry of intracellular pore are included in the GNM calculations.

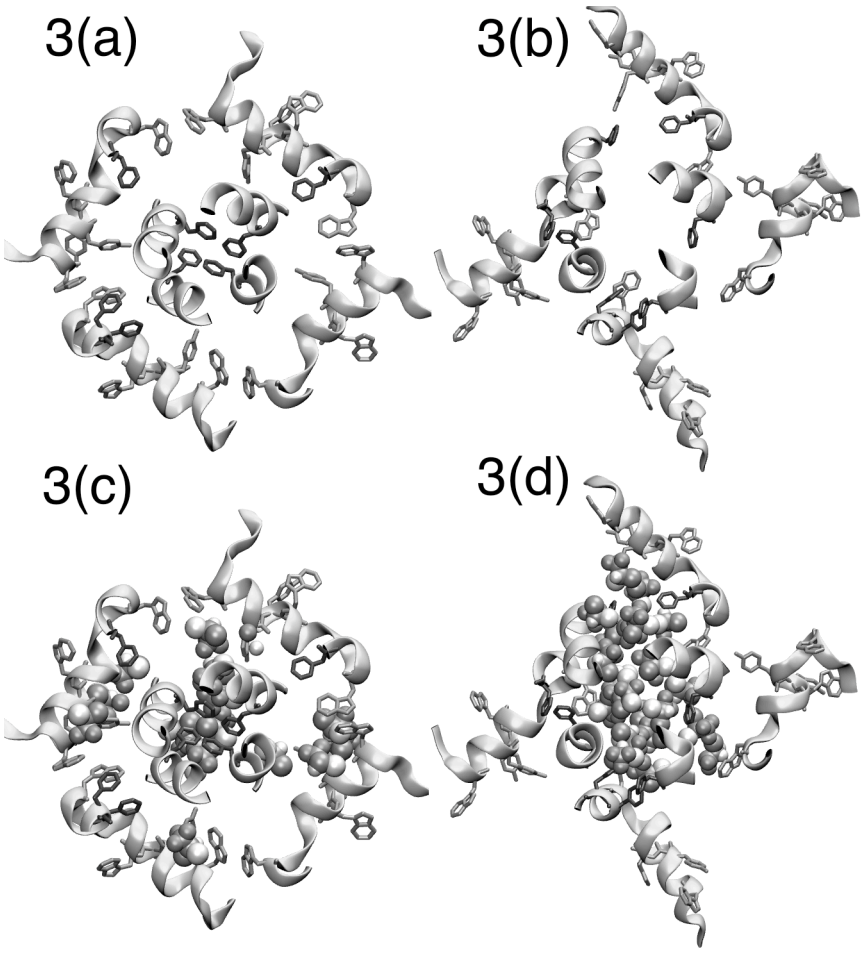
Figure 8: Interaction of aromatic residues with halothane molecules for the open structure of KirBac. The aromatic in question are PHE residues close to the pore of the open conformation, where accumulation of halothane molecules was observed in the MD simulations, as shown in Fig.3. S_N is the angle between the normal to the bilayer and a vector drawn perpendicular to the aromatic ring of PHE.



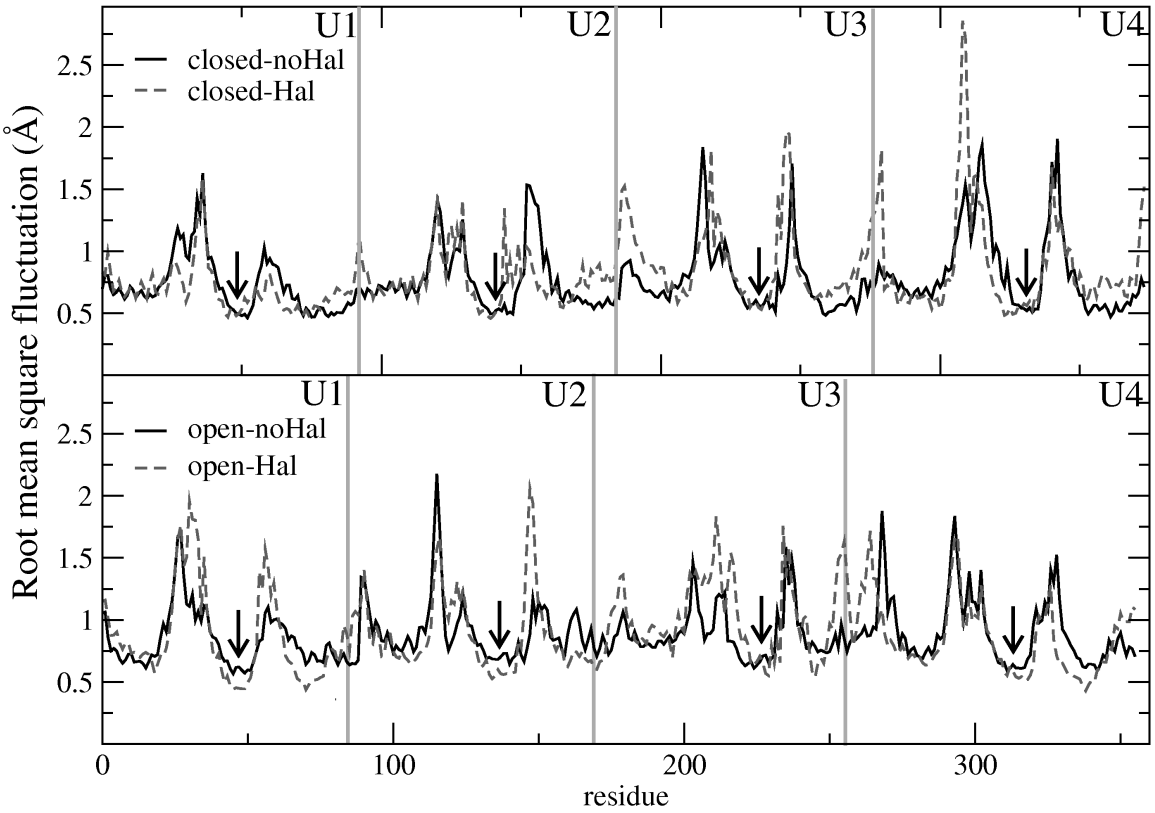
Vemparala et al_Figure 1



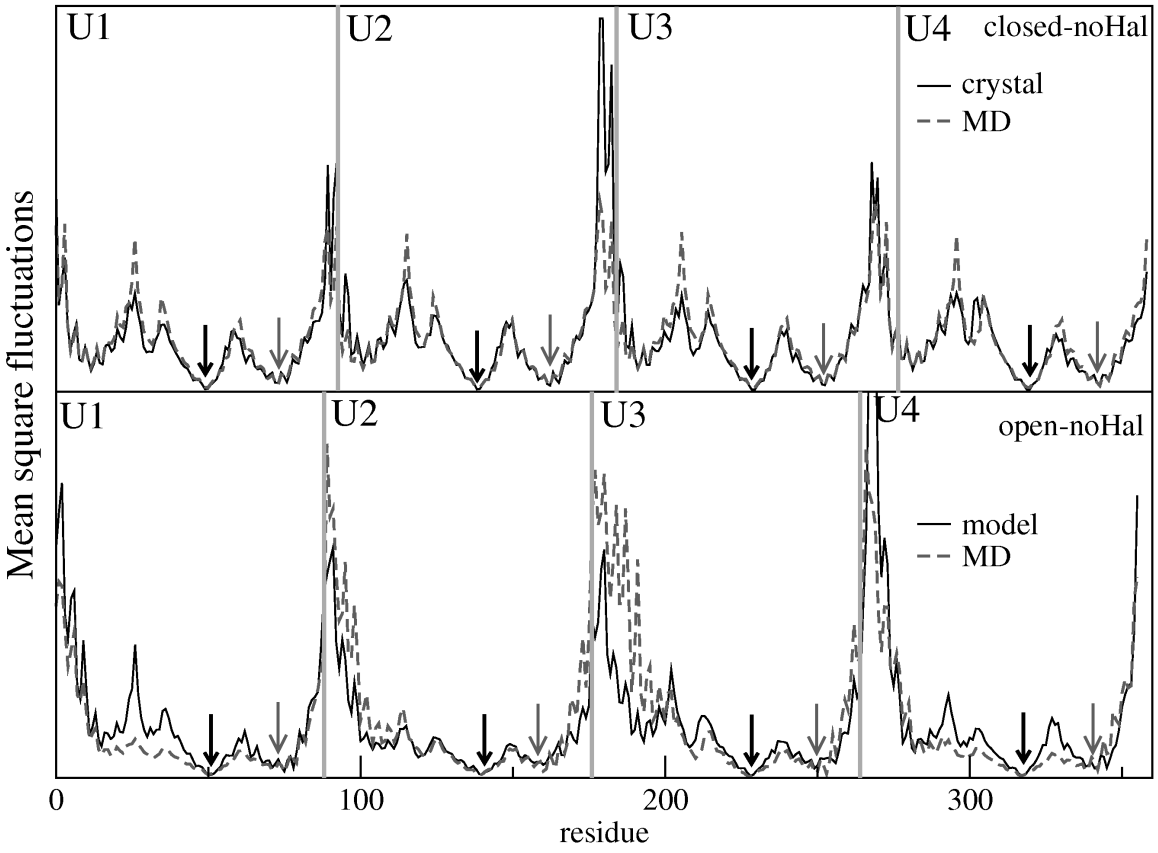
Vemparala et al_Figure 2



Vemparala et al_Figure 3



Vemparala et al_Figure 4



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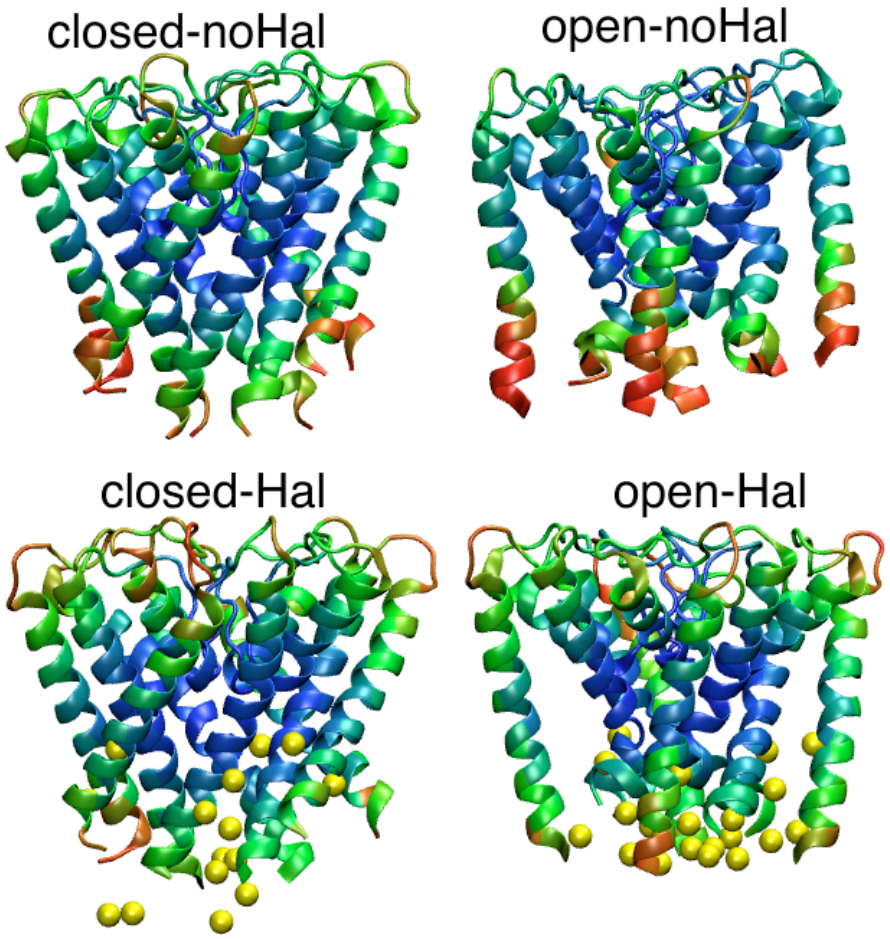
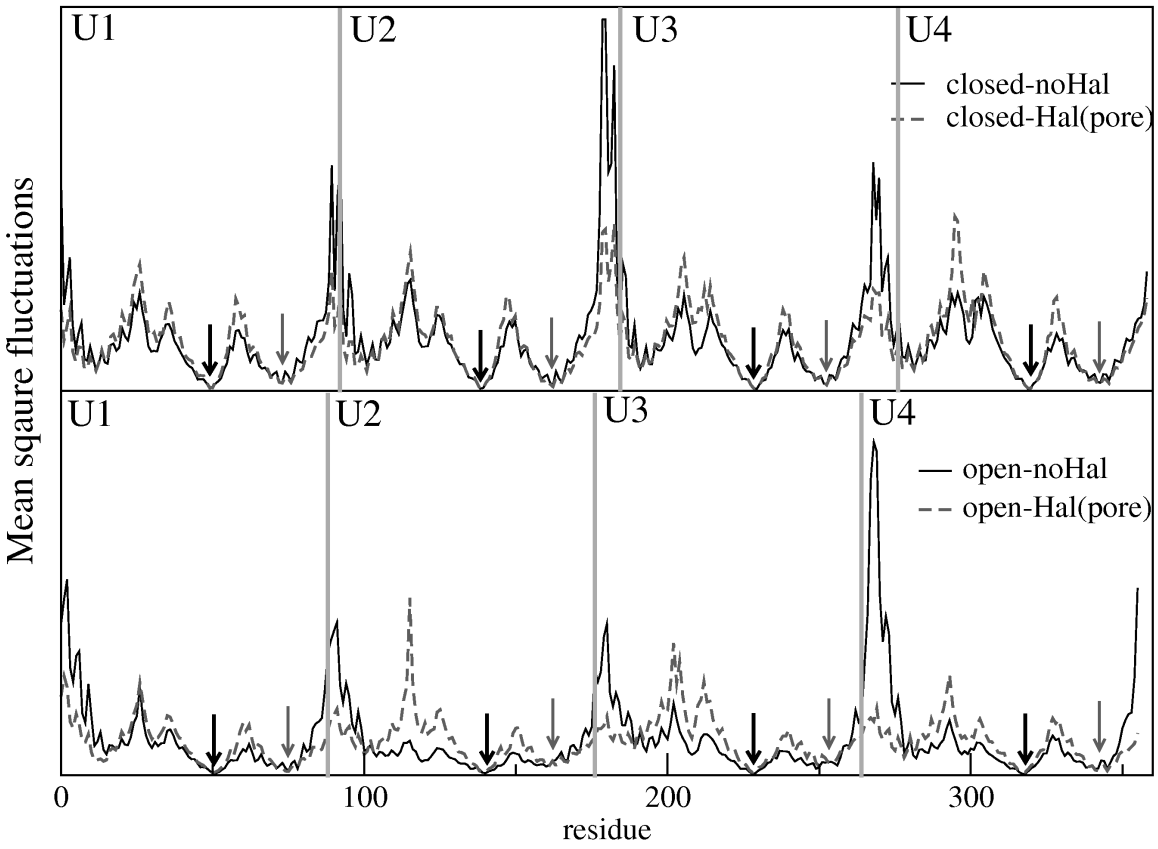
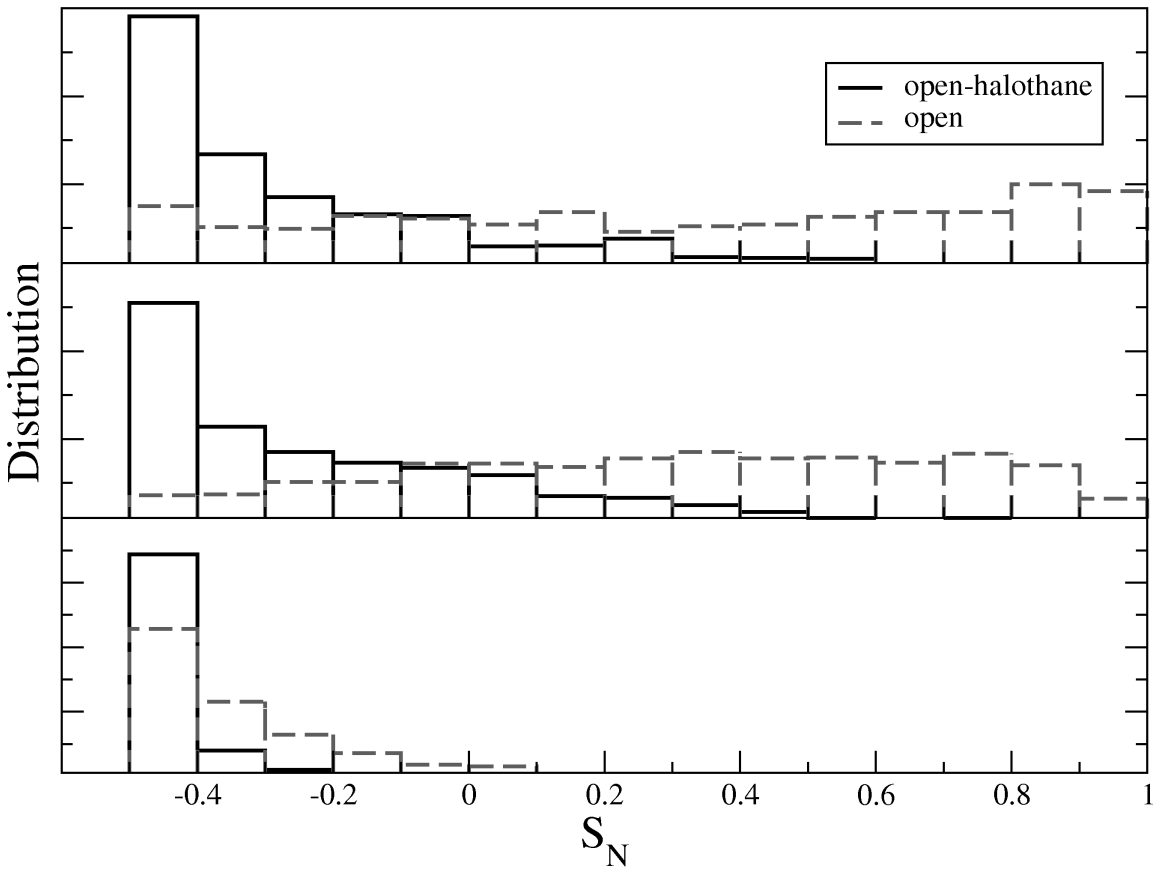


Vemparala et al_Figure 6



Vemparala et al_Figure 7



Vemparala et al_Figure 8