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Computer Simulation of Transport of Small Molecules through a Gas Channel

Embedded in a Phospholipid Bilayer

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ABSTRACT

A series of molecular dynamics simulations are performed to study the transport of ammonia and carbon dioxide through a hydrated POPC (palmitoyloleoylphosphatidylcholine) bilayer and through bacterial ammonia transporter protein (AmtB) embedded in the lipid bilayer. The diffusion coefficients of the solutes in the two different systems are calculated to compare the rate of transport of the solutes through the different types of biological membranes. The solute molecules in the POPC bilayer system are allowed to diffuse from either the interior region of the lipid bilayer or the interface between the lipid bilayer and the bulk water phase. Diffusion coefficients of ammonia are on the order of $10^{-5}$ cm$^2$/s, while those of carbon dioxide are on the order of $10^{-6}$ cm$^2$/s, indicating the size dependency of the solute in transport through the hydrated lipid bilayer. Diffusion coefficients of ammonia and carbon dioxide through the AmtB channel protein are on the order of $10^{-8}$ cm$^2$/s. The retardation of the rate of transport through the channel is attributed to the restricted displacement of the solutes in the AmtB channel protein.

The preferential association between the solutes and nitrogen atoms in the histidine residues of the AmtB channel protein is investigated by radial distribution function (RDF) analysis. The RDF results indicating strong association between ammonia and nitrogen atoms of the histidine residues supports evidence that the substrate for the AmtB protein is ammonia.
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1. INTRODUCTION

*Transport through cell membranes with molecular dynamics simulations*

Cell membranes form the boundary around a cell and allow it to maintain a proper environment for the life of the cell. The current understanding of the organization of the cell membranes is based on the fluid mosaic model of lipids and proteins [1]. Phospholipids are one of major constituent elements of the cell membranes. They are noncovalently linked by hydrophobic interactions to form the bilayer around the cell. Most of the cell membranes contain approximately 50% by weight of phospholipids. These lipids act as a formidable barrier to water-soluble substances [2]. Together with lipids, several types of proteins are present at the lipid bilayer surface and within its interior. Proteins floating like a raft on the lipid bilayer are called peripheral proteins, while proteins spanning the bilayer from one side to the other are called integral proteins. The peripheral proteins are able to act as surface receptors that recognize and bind molecules present in the extracellular environment. The integral proteins embedded within the bilayer have functions of transporting molecules from one side to the other and blocking passage into the cell for incompatible molecules.

One of the challenging problems in membrane physiology is to identify the mechanism of transport of small nonelectrolyte molecules across the cell membranes. Originally, some physiologists believed that small lipid-insoluble molecules crossed the cell membranes mainly by using small nonselective pores within the lipid bilayer [3]. However, specific transport proteins for the movement of vitally important small lipid-insoluble molecules, such as water and urea, have been identified within the last decade [4].

The types of the transport mechanisms of small lipid-insoluble molecules through the lipid bilayer are categorized by energetics and the use of a specific carrier. For passive
diffusion, some small uncharged molecules having sufficient thermal energy can enter and
cross the lipid bilayer along the concentration gradient. In this case, the driving force for the
diffusion is the concentration gradient, which renders molecules to move from an area of high
concentration to that of low one. Ions and small hydrophilic molecules can move into and out
of cells through transmembrane (TM) proteins by facilitated diffusion. Channels or pores are
created as a means of a simple form of facilitated diffusion that provides a specialized transport
route. Transport proteins are used in a complex facilitated diffusion in which small solute
molecules combine with a transporter protein dissolved in the cell membranes. Active
transport is known as the accumulation of substances within the cell or the exclusion of others
present in the environment from the cell against the concentration gradients of the transported
substances [5]. Active transport requires the expenditure of chemical work to an energy-
consuming reaction, usually hydrolysis of adenosine triphosphate (ATP). There are two types
of active transport, primary and secondary active transport. Primary active transport is when
ATP-dependent membrane pumps are used to transport substances against a gradient, requiring
the direct expenditure of energy. Secondary active transport allows a substance to move
against an electrochemical gradient because the substance moves down its own concentration
gradient.

The passive diffusion of small molecules or ions across the lipid bilayer can occur
through aqueous pores or ion channels. TM proteins in the cell membranes form aqueous
pores connecting the extracellular space with the internal fluid of the cell or cytosol. Ion
channels are specifically designated to transport inorganic ions such as $\text{K}^+$ and $\text{Na}^+$ across the
cell membranes. Due to the narrowness of these channels, only small molecules and ions can
effectively diffuse through, protecting the cell from larger and incompatible molecules. One
important difference between aqueous pores and ion channels is that aqueous pores remain open, but ion channels are gated and open and close in response to specific stimuli [2].

A variety of methods has been performed to study the structures and dynamics of the cell membranes. Current methodologies on cell membrane research have offered rather incomplete atomic or molecular details due to the lack of sufficient experimental data under physiological conditions. However, the availability of powerful computers and fast computing algorithms has shed light on computer simulation methods using a model system in an attempt to overcome the shortcomings of the experimental methods. One of the most common computer simulation techniques used in molecular modeling is Molecular Dynamics (MD). The use of MD has been greatly enhanced since the first Molecular Dynamics simulation of a cell membrane model [6] and the availability of general-purpose force fields for water, proteins, and DNA, such as AMBER [7] and CHARMM [8].

In a MD, atoms in the system are treated like in classical mechanics. A typical functional form of a force field is:

\[
E = \sum_{\text{bonds}} \frac{k_b}{2}(l_i - l_{i0})^2 + \sum_{\text{angles}} \frac{k_i}{2}(\theta_i - \theta_{i0})^2 + \sum_{\text{dihedrals}} \frac{k_i}{2}(1 + \cos \{n_i(\phi_i - \phi_{i0})\})
\]

\[
+ \sum_{j=1}^{N} \sum_{j=1}^{N} \left(4\epsilon_{ij} \left[ \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] + \frac{q_i q_j}{4\pi \epsilon_0 r_{ij}^2} \right)
\]

(1.1)

where \( k_b, k_i, \) and \( k_{\phi_i} \) are force constants for bonds, angles, and dihedrals, \( l_{i0}, \theta_{i0}, \) and \( \phi_{i0} \) equilibrium values for bond lengths, angles, and dihedral angles, \( n_i \) the dihedral multiplicity, \( \epsilon_{ij} \) and \( \sigma_{ij} \) Lennard-Jones parameters, \( r_{ij} \) the distance between atom \( i \) and \( j \),
$q_i$ and $q_j$ the partial charges on atom $i$ and $j$, and $\varepsilon_0$ the electrical permittivity. Using this potential function, the equation of motion for the atoms in the system can be solved by calculating the forces on all atoms and integrating with respect to time. The result of the calculation is a trajectory including the coordinates and velocities of all atoms at any of the integration steps. This procedure makes MD simulation a powerful technique to study cell membrane systems on the atomic level.

Nevertheless, MD simulation has limitations in representing real systems through numerical calculations. The functional form in Equation 1.1 requires a large number of parameters for equilibrium values of bonds, angels, dihedrals, and force constants and for van der Waals interactions and partial charges. Many of these parameters can be obtained through spectroscopic experiments or quantum mechanical calculations. This fact means that the accuracy of the result from MD simulations would be dependent upon that of the chosen force field and its parameters rather than characteristics of the systems. In many cases, the parameterized values cannot guarantee good results on the behavior of atoms and molecules in the given system. Secondly, the classical treatment of atoms present in the system makes MD simulation unsuitable for treatment of chemical reactions without employing quantum mechanical calculations. Finally, the spatial and temporal scales that are required to study the structures and dynamics of the cell membranes are considerably out of reach of currently available MD simulation methodologies. In practice, the size of a model membrane system consists of approximately 100 – 200 lipid molecules and the length of time is limited up to a few tens of nanoseconds in fully atomistic simulations. These scales are relatively close to the initial configuration because the rotational and translational motions of lipids are too slow.
to sample accurately in a few nanoseconds [9]. This is also true for the slow process of permeation of small molecules through a lipid bilayer [10].

It is expected to establish a good membrane channel model including lipid bilayers, proteins, and water molecules to study more realistic biological systems with MD simulation methodologies. While there are limitations and the need to validate MD simulations, MD provides trajectories that are advantageous in studying the most detailed structures and dynamics of the biological systems on the atomic level. Various types of lipid-protein systems have been studied such as membrane spanning channels. The ideal prototypical system of the membrane spanning channels is the gramicidin A embedded in fully hydrated dimyristoylphosphatidylcholine (DMPC) bilayer [16,17]. The microscopic model is comprised of 16 DMPC, one gramicidin A dimer channel protein, and 649 water molecules. The MD simulation study proved that the method of constructing the microscopic model of gramicidin A is in excellent agreement with available experimental data by showing that the order parameters and backbone conformations can be reproduced.

Since a model represents the structures used in MD simulations, the results from the simulations need to be validated by corresponding experimental data. If there is good agreement between the simulation results and the experimental data, the model can be employed to interpret phenomena that cannot be studied by existing experimental techniques. A few experimental techniques used for the study of the structures and dynamics of lipid bilayers include X-ray and neutron diffraction [11], Nuclear Magnetic Resonance (NMR) spectroscopy [12,13], and fluorescence techniques [14,15]. Among these, $^2$H-NMR of deuterated lipids provide a way to calculate order parameters accurately. These order parameters can be calculated from simulations and then compared to the experimental data.
Statement of purpose

Cell membranes are selective barriers to gas molecules in biological systems. The factors affecting the rate of transport of gas molecules through the cell membranes are rather unclear due to the difficulty of accurately measuring gas fluxes. A number of approaches have been introduced to describe mechanisms and functions of the transport of small molecules across the cell membranes both in lab experiments [18-21] and computer simulations [10,22-26]. In this work, two types of membrane models, a hydrated phospholipids bilayer and a channel protein embedded in the lipid bilayer, were constructed to study energetics and the transport process of ammonia and carbon dioxide with MD simulations. Most small nonelectrolyte molecules can cross the cell membranes either through specific channels or by partitioning into the lipid bilayer and diffusing through the hydrophobic region of the lipids [27]. Typical small molecules are water (H\textsubscript{2}O), ammonia (NH\textsubscript{3}), carbon dioxide (CO\textsubscript{2}), and oxygen (O\textsubscript{2}). Water is the most abundant substances in the cells, ammonia is transformed into acidic excretions in the form of ammonium cation (NH\textsubscript{4}\textsuperscript{+}) as a product of cellular metabolic processes, and carbon dioxide and oxygen are essential molecules in cellular respiration. Among these small nonelectrolyte molecules, ammonia and carbon dioxide are the solute molecules allowed to diffuse through the model systems. The choice of ammonia and carbon dioxide is motivated by the characteristic behaviors that they are readily dissolved in water. Especially, the transport of ammonia and carbon dioxide is restricted in certain biological membranes, requiring the presence of selective channels to control the flux of those small nonelectrolyte molecules [28,29]. Recently, life scientists have resolved problems in
determining the mechanisms of the movement of ammonia and carbon dioxide molecules that cross the cell membranes through membrane proteins. The evidence supports that ammonium/methylammonium transporter (Amt) proteins act as gas channels for ammonia, which may play a role in ammonia transport because diffusion through the lipid bilayers is too slow for physiological needs of the cells [30,31]. The gas channels can also be used as a molecular target for regulating the pathways of ammonia [32]. The structure of the Amt protein and the mechanism of gas transport are commonly found in the members of the Rh superfamily in eukaryotic cells. This suggests the presence of Rh-related proteins. The Rh blood group antigens of erythrocyte membrane are the best known Rh proteins and are present ubiquitously in human organs including the kidney and brain [36]. The evidence for the role of the Rh proteins in carbon dioxide transport is provided by several studies [33,34]. By studying a green alga, studies were able to conclude that the physiological substrates for Amt and Rh proteins are ammonia and carbon dioxide, respectively [35].

The direct applicability of the results obtained from the current project to clinical or pharmaceutical researches is conjecture. However, the impact of the research results may contribute to the construction of database of drug candidates or drug delivery systems (DDS).

There are many general mechanisms for drug uptake. Among a number of routes for effective drug actions, oral administration is the most convenient and acceptable way for the treatment of disease [26]. In certain cases, intravenous infusion has to be carried out to achieve specialized pharmacologic and therapeutic goals by making 100 % of the drug available in the blood [37]. Once the drug is in the blood stream, the initial step for drug action requires uptake into the cells across the cell membrane to reach the biological target sites or tissues. The drug actions depend upon a number of factors such as the rate of removal
of drugs, bioavailability of drugs, and the affinity of the transport process across the cell membranes [37]. The delivery of both drugs and physiologically important molecules is influenced by the extent of interaction with the target sites and through specific receptors that permit the substances into or out of the cells.

Based on the microbiological background, a methodology to interpret data from the MD simulations is to focus on two objectives. The first objective is to consider energetics of the model systems. The trajectories from MD runs are used to calculate diffusion coefficients of ammonia and carbon dioxide from the mean-square displacement (MSD) and the Einstein relation [38]:

\[
2dD = \lim_{t \to \infty} \frac{\langle |r(t) - r(0)|^2 \rangle}{t}
\]

(1.2)

where the numerator is the MSD of the diffusion at time \( t \), and \( d \) is 2 for 2-dimensional diffusion and 3 for 3-dimensional diffusion. The diffusion coefficients from the two models of the hydrated lipid bilayer and the membrane channels are compared to see how the gas channel plays a role in the transport of the diffused species. The overall conduction of the solute molecules is simulated only in approximately 20 Å long region within the Amt protein channel. The deprotonation of ammonium cation around the channel vestibule cannot be simulated due to the inherent drawback of the employed force field, which is not able to handle chemical reactions. The second objective is to focus on the structural correlations of the model systems. The radial distribution functions (RDFs) between the \( \epsilon \) and \( \delta \) nitrogen of the two conserved histidine residues in the Amt protein and the solute molecules are obtained to
investigate the preferential association between them. The images of the available free volume within the channel before and after the MD simulations and the expected paths of the solute molecules are generated by the execution of a series of scripts.
2. LITERATURE REVIEW

Mep/Amt/Rh proteins

In higher animals and humans, the preferred nutrition is accomplished through the synthesis of amino acids and the byproduct of the metabolism of nitrogen results in excretion of ammonium ions [39]. It is reported that TM fluxes of ammonia and ammonium ion have been studied mostly in the mammalian kidney, where excretion of ammonium ion constitutes the major component of the excretion to maintain pH balance, and in the brain where a flux of ammonium from neurons and neuroglia cells is found in most nervous tissues [40]. Thus, high levels of exogenous ammonium may cause metabolic acidosis and renal failure [41], and have been linked to Alzheimer’s disease [42].

Ammonia is rapidly protonated to yield ammonium cation, having a pKₐ of 9.25. Under a normal physiological condition of neutral pH, more than 99% of the ammonia will be in the ionic form [43]. As a result, both the diffusion of ammonia across the cell membranes and the transport of ammonium ion through specific carriers can potentially occur in biological systems [40,44]. The first establishment of the properties of the ammonia transport systems was proposed in the fungi Penicillium chrysogenum and Aspergillus nidulans [45]. The genetic approaches with Escherichia coli to identify potential bacterial ammonium transporter (AmtB) have been made, however, they were unsuccessful [46]. The cloning of two genes from yeast Saccharomyces cerevisiae Mep1 and plant Arabidopsis thaliana Amt1 contributed to the identification of the first two ammonia transporter proteins [47,48]. The two genes that encode ammonium transport proteins have common topologies of an external amino terminus, an internal carboxyl terminus, and 11 TM spans [49]. The characterization of AmtB
catalyzed the search on the transporter homologues in other organisms. A bacterial homologue from the isolation of AmtB in *Corynebacterium glutamicum* works much like an ammonium transporter [50] and the presence of it gives rise to the possibility of finding related proteins in higher animals or humans. The assumption led to a finding on the mammalian homologues. In an attempt to test the capability of ammonium transport in ammonium-uptake deficient yeast, the homologues were identified as RhAG, a Rh blood group antigen, found in erythrocytes and the kidney and liver [51,52]. From experimental evidence supporting the cell membrane of erythrocytes is permeable to ammonia but not to ammonium cation [53] and the concentration of cytoplasmic ammonium is maintained higher [54], facilitated diffusion of ammonia molecules along their concentration gradient would explain the uptake and efflux capabilities of RhAG [46]. The topology of Rh proteins is distinguished from Amt proteins in that it was empirically determined to fit a twelve TM helix model with both the amino and carboxyl termini on cytoplasmic side [55].

Carbon dioxide, like ammonia, is readily hydrated in water and its rate of transport is hindered in the apical membranes of epithelial cells exposed to hostile environments [29]. The molecular mechanisms of the transport of carbon dioxide through biological membranes have been investigated in a number of studies [33,34]. The Rh proteins and aquaporin1 (AQP1) water channels were proposed to be gas channels for carbon dioxide [56], with evidence which concludes an involvement of a protein in carbon dioxide transport through the red blood cell membrane [57]. The research group of Soupene *et al.* [34] studied expression of the *RH1* gene of the microbe *Chlamydomonas reinhardtii* and reached the conclusion that the Rh protein appears to be a gas channel for carbon dioxide with its primary biochemical function to facilitate diffusion of carbon dioxide.
The retarded growth of either AmtB defective *E. coli* or Mep defective yeast supports evidence that AmtB or Mep is demanded if the pH-dependent concentration of uncharged ammonia drops below a critical value, e.g., 1mM [58,59]. This leads to a fact that either AmtB or Mep mediates the transport of ammonia with respect to the change of pH and concentration of ammonia. The different mechanisms exist for the Rh proteins in mammalian cells, where membrane potentials are smaller and controlled by Na\(^+\)/K\(^+\) pumps [40]. The change of the transport in the Rh protein RhAG with the radioactive analogue of ammonium (\(^{14}\)C-methylammonium) was studied and the result showed that transport increased with external pH but decreased with increasing internal pH of *Xenopus* oocytes [60]. If the systems contain real substrates that should be substituted with an analogue and different substrates present simultaneously, as in the case of ammonia and ammonium cation, the characterization of transporters and channels in model systems, however, cannot provide conclusive information on the mechanisms of transport without using electrophysiological methods [61].

**Gas channel protein**

The molecular identity of the bacterial ammonia transporter protein in *E. coli* has been identified recently with unprecedented crystallographic resolution at 1.35 Å [30]. Though the family of ammonium transporter proteins (Mep/Amt/Rh) is present in a number of domains of organisms, research on finding the molecular mechanisms and chemical identities of the transported species have yielded controversial results [45,46,54]. The current view on the family of ammonia transporter proteins is widely considered as secondary transporters that
mediate uptake of ammonium cation \([39,62,63]\). However, the view has been challenged by evidence showing that Mep/Amt proteins are channels which simply increase the rate of equilibration of ammonia across the cytoplasmic membrane \([31,33,58,59]\). The pathways for diffusion are not solely through the cell membranes of certain tissues, which have relatively low permeability to gaseous compounds such as ammonia and carbon dioxide \([29]\). In that case, they may have alternative routes to fulfill the physiological needs for the transport of the small molecules through a protein \([57]\).

The structure of AmtB consists of identical trimer, each of which has a narrow and approximately 20 Å long hydrophobic channel for only ammonia to pass through in single file form \([30]\). The trimeric structure also gives a key to how the three Rh polypeptides of red blood cells form the Rh antigen complex in the erythrocyte plasma membrane \([32,64]\). Most evidence suggest that the Rh proteins in humans can play a role of a gas channel for the conduction of ammonia and carbon dioxide across eukaryotic cell membranes to regulate body pH and ammonia toxicity in the kidney and brain \([30,33,58,59]\). Furthermore, the role of the Rh proteins in the transport of carbon dioxide is clarified by additional evidence through genetic studies with \textit{Chlamydomonas reinhardtii}, having both Amt and Rh proteins, in that the physiological substrate for the Amt proteins is ammonia and that for the Rh proteins is carbon dioxide \([35]\).

Transport of ammonia occurs through the channel located at the center of each monomer of the AmtB trimer \([30,31]\). From the periplasmic side, the two aromatic side chains of Phe\(^{107}\) and Phe\(^{215}\) control access of molecules into the channel. This region is a binding site for ammonium cation using \(\pi\)-cation interactions \([30,31]\). The effective diameter of the channel entry is 1.2 Å, and thus, the aromatic side chains of the two phenylalanine
residues should move dynamically during the course of transport [30]. Inside the hydrophobic channel, the side chains of His$^{168}$ and His$^{318}$ lie adjacent with their imidazole ring edges facing the cavity such that a hydrogen bond can be formed between the δ nitrogens [31]. Assuming neutral hydrophobic environment in the cavity, the ε nitrogens would be one acceptor and one donor function to the diffusion of ammonia through the channel [31]. Additional information from the adiabatic free energy profiles on ammonia and ammonium ion suggests that AmtB facilitates the transport of neutral ammonia while a hydrophobic pore within the channel is a barrier to the cation [65]. No significant conformational changes are observed during diffusion and this makes AmtB considered as a slowly conducting channel rather than a transporter [30], which should have flexible elements involved in translocation of solutes [32]. A quantitative approximation on the rate of transport showed that the estimated flux lies in the range of $10^1$ to $10^4$ ammonia molecules per second per channel, which is far below the rate of typical open channels operating close to the diffusion limit of $10^8$ to $10^9$ molecules per second [31]. The slow rate of the transport may be attributed to the two following reasons: structural fluctuation of the pore entry and its hydrophobic environment into the pore [31].

**Computer simulation studies on transport through biological membranes**

The choice of methods and parameters used to run computer simulations is critical to reliable results from the model systems. The studies of biological membranes can be divided in two main categories, methodology and application [66]. The methodology has focused on the technical issues of empirical force field parameters [67], methods to control temperature
and pressure [24,68], and algorithms for calculation of long-range interactions [69] to name a few. The application has dealt with systems of biophysical, biochemical, and pharmaceutical interests [70]. For example, studies in transport of small or drug candidate molecules through biological membranes have been prevalent with MD simulations, giving us insight into the transport on the atomic scale.

The diffusion of small molecules through lipid bilayers is one of the essential transport phenomena across biological membranes. The first atomic level MD simulation of solute diffusion through lipid bilayers was performed in 1993 [71]. The model system used in the study is a fully hydrated dimyristoylphosphatidylcholine (DMPC) bilayer in a liquid-crystalline ($L_a$) phase with benzene molecules as solutes at 320 K. A total of four simulations were conducted in the study. Three of them contained one benzene molecule placed at different positions in the DMPC bilayer while the length of simulation time was varied from 525 ps to 1100 ps, and the other was with four benzene molecules for 1100 ps in the lipid bilayer. The rate of diffusion was calculated from the slope of the MSD at long times in the region of the Einstein diffusion and was found to be on the order of $10^{-6}$ cm$^2$/s. The authors found that there is no evidence for solute molecules to have a preference for a particular region of the bilayers, while the rate of diffusion in the interior region of the lipid bilayer is two or three times faster than that in the headgroup region of the lipid bilayer. As a consequence, the diffusion mechanism of benzene molecules in the DMPC bilayer was proposed to be jump or hopping of the benzene molecules among the available free volume in the lipids [72], similar to the diffusion in soft polymers [73].

As a sequel, the study on the effect of temperature and the preferred locations of the benzene molecules on the rate of diffusion was published in 1995 [74]. The same membrane
model of fully hydrated 36 DMPC lipids was used with four benzene molecules as solutes in each model. The temperature in the study was changed from 310 K to 340 K in steps of 10 K. The regional preference of the benzene molecules at different temperatures supports the fact that the distribution of free volume in the lipid bilayer is concentrated at the center of the bilayer at lower temperatures, however, free volume is diffused toward the headgroup region at higher temperatures. This fact conclusively shows that the rate of diffusion depends on the region within the bilayer. To calculate the rate of diffusion, a plot of the logarithm of the MSD vs. logarithm of time was used to determine if the diffusion reached equilibrium [75]. The log-log plot gives a slope of 1 when in the region of Einstein diffusion, a slope of 2 for non-Einstein diffusion of ballistic motion, and 0.5 for anomalous diffusion [76]. The two successive works by Bassolino-Klimas et al. [71,74] on the diffusion of benzene molecules through a DMPC bilayer lead to a conclusion that lipid bilayers are significantly different from bulk hydrocarbons and would exist in a more realistic heterogeneous phase.

As an extension of the two previous diffusion studies, the benzene molecule as a solute was substituted with a drug (nifedipine) analogue molecule in the same system to obtain insight into the interactions of a drug molecule within the membrane and its diffusion mechanism [77]. It was found that the rate of diffusion of the nifedipine analogue molecule has no considerable change with respect to the locations within the bilayer. This is due to the larger size of the nifedipine analogue, and thus it is able for the nifedipine analogue to follow the hopping diffusion mechanism observed in the benzene molecules. In the interior region of the lipid bilayer, the diffusion coefficients were found to be on the order of $10^{-6}$ cm$^2$/s, about one-third of that for benzene molecules. The preferred orientation of the nifedipine analogue near the water interface becomes significant, indicating an increase of the hydrogen bonding of
the nifedipine analogue with the lipid headgroup molecules and water. In terms of the size effect of solutes on diffusion, another study with methanol and propanol as solutes in a DMPC bilayer emphasized that the size of solute is critical on the rate of diffusion within the bilayer [78]. This was elucidated by showing that the smaller solutes diffuse faster and the phenomenon is even more pronounced in the region of headgroup of the lipid bilayer.

The permeation of small molecules through lipid bilayers has been also of considerable interest on the transport study of the biological membranes. The process of permeation in the membrane is affected by both diffusivity and solubility of the penetrants into the membrane [79]. The study on permeation of water [10], and ammonia and oxygen [22] across a lipid bilayer was reported in two separate papers, using a fully hydrated dipalmitoylphosphatidylcholine (DPPC) bilayer model. The authors proposed a four-region model based on the characteristic behavior of the lipids in the membrane to account for the heterogeneity of it. The overall rate of permeation was calculated by computing the local diffusion coefficients as well as the solubility of the small molecules in the framework of the four-region model. One of the important conclusions resulting from the work is that the diffusion coefficients and the solubility of the small molecules are strongly dependent upon the location within the lipid bilayer. Therefore, the proposal of the four-region model is supported by the conclusion and explains the oversimplified nature of the lipid bilayers from a homogeneous solubility-diffusion model [80]. Another conclusion is that the diffusion is fastest in the interior lipid bilayer of low lipid density and the rate-determining step is the diffusion through the dense part of the fatty acid chain. The calculated diffusion coefficients at 350 K are on the order of $10^{-4}$ cm$^2$/s in the middle of the lipid bilayer and $10^{-5}$ cm$^2$/s in the lipid headgroup region. The latter paper extended the study of the permeation of the small
penetrants with the effects of hydrophobicity, size, and shape [22]. In permeation of hydrophobic molecules, as in the case of oxygen, the membrane aids in permeation rather than slow it down. The authors believed that this effect is mainly due to the large solubility of hydrophobic penetrants in the membrane. The solubility and the diffusivity in the region of the highest lipid density depend on the size of the small penetrants. This is shown by the analysis of the free volume and accessible holes in the lipid bilayer [81], and thus provides evidence for the hopping mechanism in the diffusional part of the permeation process. The highest lipid density region also functions in discriminating between spherical penetrants and elongated penetrants that yield relatively high permeation rate in it.

Recently, the studies on permeation of small molecules through the membranes have expanded further with small organic compounds in an attempt to lay a scheme for rational drug design [82,83]. The small organic compounds representing the most common functional groups are acetamide, acetic acid, benzene, ethane, methanol, methylacetate, methylamine, and water. The same simulation methods used in the previous permeation studies by Marrink et al. [10,22] were employed for these MD simulations. The purpose of the first study by Marrink et al. was to calculate the equilibrium and dynamic properties of the solutes in the lipid bilayer to investigate the permeation behavior of the solutes in DPPC bilayer [82]. The equilibrium property can be explained by the free energy profiles. The general trend is that solute free energy increases as going into the lipid bilayer from the water phase. For the hydrophilic molecules, the free energy continuously increases on moving toward the interior lipid bilayer. However, the hydrophobic molecules show the opposite behaviors, which are observed in the two previous permeation studies by Marrink et al. The dynamics property can be described by diffusion coefficients. The diffusion coefficients of the solute molecules
are generally decreased for all the solutes as going into the interior lipid bilayer, while there is a slight increase of them due to the available free volume at the center of the lipid bilayer.

The purpose of the second study by Marrink et al. was concentrated on the correlation between the transport phenomena through the lipid bilayer and the physical properties of the solutes, such as size and cross-sectional area [83]. The diffusion coefficients within the lipid bilayer show less size dependence than those in the bulk water phase. Instead, the partitioning of the solute into the lipid bilayer significantly depends on the solute size. In addition, the correlation between diffusion and two different size factors of the solutes, i.e. the molecular volume and the cross-sectional area, has been investigated in polymer solvents [84]. In the case of these eight organic compounds, the dependence of the solute cross-sectional area on diffusion is approximately as large as twice that of the molecular volume.

The studies of biological membranes have begun to incorporate proteins in the lipid bilayer to mimic more realistic biological membrane systems. One of the best experimentally characterized membrane-protein system is gramicidin A channel embedded in DMPC [16,17]. Gramicidin A forms a right-handed head-to-head dimer spanning the membrane with an open cylindrical pore that conducts small univalent cations [85]. The construction approach suggested by Woolf and Roux can be used to generate starting configuration for membrane protein in a lipid bilayer and achieve a breakthrough in MD simulation studies of lipid bilayers with proteins. The starting configurations were selected from a set of preequilibrated and prehydrated DMPC molecules to match as closely as possible to available experimental data. The randomly selected sixteen DMPC molecules from the library of preequilibrated and prehydrated DMPC molecules were placed around the gramicidin A through rotation around the z-axis and translation in the xy-plane in hexagonal symmetry. Each leaflet of the bilayer
contained eight DMPC molecules and a gramicidin A monomer. The lipid bilayer has no significant contribution to the conformational change of the gramicidin A with the average root-mean-square deviation (RMSD) of 1.2 Å throughout the 500 ps trajectory. The channel protein effectively increases the ordering of neighboring lipids, as stabilized by the tryptophan (Trp) side chains.
3. COMPUTER SIMULATION METHODOLOGY

Equation of motion

In the classical treatment of atoms in MD methods, the positions and velocities of the atoms are calculated by the Newtonian equation of motion as:

\[-\nabla E(r) = m \frac{d^2 r}{dt^2}\]  \hspace{1cm} (3.1)

where \( E \) is the potential energy, \( r \) the position, and \( m \) the mass of an atom. The differential equations of motion are integrated using algorithms of finite difference method [38]. The force \( F \) acting on each atom is determined from the gradient of the potential function. From the position \( r \), the force \( F \) at time \( t \) is calculated for all the pairs of the atoms in the system and from the force \( F \), the acceleration \( a \) is calculated. The new position \( r(t + \delta t) \) and the new velocity \( v(t + \delta t) \) are calculated after the time step \( \delta t \). The Verlet algorithm [86] and its modifications are now the most commonly applied method for integrating the equations of motion in MD simulations [38]. For example, the velocity Verlet algorithm [87] gives the atom positions, velocities, and accelerations simultaneously using the following equations:

\[ r(t + \delta t) = r(t) + \delta t v(t) + \frac{1}{2} \delta t^2 a(t) \]  \hspace{1cm} (3.2)

\[ v(t + \delta t) = v(t) + \frac{1}{2} \delta t [a(t) + a(t + \delta t)] \]  \hspace{1cm} (3.3)
The typical restriction for the time step is one order of magnitude smaller than the fastest motion in the system [38].

**Potential energy function**

A functional form in Equation 1.1 can be used to calculate the interactions among all atoms present in the system of interest. The functional form is called the force field and it approximates the real potential energy from the numerous terms in the potential energy function using empirically determined sets of parameters possessing equilibrium values for the motions of atoms. However, the precise form of the force field has to be varied with respect to the system and the purpose of study.

In practical MD simulations of biological molecules, CHARMM is the most common force field. Its potential energy is computed by the sum of the contributions from bond stretching, angle bending, dihedral torsions, electrostatic and van der Waals interactions [8]:

\[
E = \sum E_{\text{bonds}} + \sum E_{\text{angles}} + \sum E_{\text{dihedrals}} + \sum E_{\text{electrostatic}} + \sum E_{\text{vdW}}
\]  

(3.3)

The bond and angle contributions are described as harmonic potentials that increase the potential energies as the bond length \( l_i \) and the angle \( \theta_i \) deviate from the equilibrium values \( l_{i,0} \) and \( \theta_{i,0} \), respectively:
\[ \sum E_{\text{bonds}} = \sum_{i=1}^{N} k^b_i (l_i - l_{i,0})^2 \]  
\[ \sum E_{\text{angles,harmonic}} = \sum_{i=1}^{N} k^\theta_i (\theta_i - \theta_{i,0})^2 \]  

where \( k^b_i \) and \( k^\theta_i \) are the force constants for bonds and angles, respectively. The CHARMm force field also includes an additional cross-interaction term for the angle bending motions. The angle bending is modeled using the Urey-Bradley potentials accounting for the van der Waals interactions between the nonbonded atoms 1 and 3:

\[ \sum E_{\text{angles, Urey-Bradley}} = \sum_{i=1}^{N} k_{UB}^i (u_i - u_{i,0})^2 \]  

where \( u_{i,0} \) is the equilibrium distance and \( k_{UB}^i \) the force constant. The torsional terms are described as:

\[ \sum E_{\text{dihedrals}} = \sum_{i=1}^{N} k^\phi_i [1 + \cos(n_i \phi_i - \delta_i)] \]  

where \( k^\phi_i \) is the force constant, \( n_i \) is the dihedral multiplicity, \( \phi_i \) is the dihedral angle, and \( \delta_i \) is the phase angle. An additional term is incorporated in the CHARMm force field to
describe atom motions that bend out of plane:

\[
\sum E_{\text{improper}} = \sum_{i=1}^{N} k_{ij} (\psi_i - \psi_{i,0})^2
\]  

(3.8)

where \( \psi_i \) is the improper torsional angle, and \( k_{ij} \) is the force constant. The nonbonded interactions are calculated between the atom pairs \( i \) and \( j \) separated by at least three bonds. The electrostatic static interactions are modeled using the Coulombic potential:

\[
\sum E_{\text{electrostatic}} = \sum_{i=1}^{N} \sum_{j=1}^{N} \frac{q_i q_j}{4\pi\varepsilon_0 r_{ij}}
\]  

(3.9)

where \( q_i \) and \( q_j \) are the partial charges on atom \( i \) and \( j \), \( r_{ij} \) is the separation distance, and \( \varepsilon_0 \) is the electrical permittivity. The van der Waals interactions are modeled using a Lennard-Jones (LJ) potential:

\[
\sum E_{\text{vdW}} = \sum_{i=1}^{N} \sum_{j=i+1}^{N} \varepsilon_{ij} \left[ \left( \frac{R_{\text{min},ij}}{r_{ij}} \right)^{12} - 2 \left( \frac{R_{\text{min},ij}}{r_{ij}} \right)^{6} \right]
\]  

(3.10)

where \( \varepsilon_{ij} \) is the well depth at the energy minimum and \( R_{\text{min},ij} \) is the distance between atom \( i \)
and $j$ at the energy minimum. When the interactions between the different types of atoms are necessary, the CHARMm force field employs mixing rules to determine the LJ parameters in the following way:

\[ R_{\text{min},ij} = \frac{R_{\text{min},ii} + R_{\text{min},jj}}{2} \]  \hspace{1cm} (3.11)

\[ \epsilon_{ij} = \sqrt{\epsilon_{ii} \epsilon_{jj}} \]  \hspace{1cm} (3.12)

As shown in the above equations, the force field requires a number of parameters to describe the interactions of atoms adequately. However, the parameters are empirically determined. Several sets of parameters including equilibrium values, force constants, partial charges, and the LJ parameters are obtained from spectroscopic experiments and \textit{ab initio} calculations. The parameterization is iteratively performed until the parameterized force field describes the behaviors of the atoms in the simulated system close enough to those in actual systems. Occasionally, the parameterizations are aimed to avoid unfavorable behaviors during a simulation.

\textit{Periodic boundary conditions}

Periodic boundary conditions (PBCs) are usually employed in order to diminish the finite size effect at the boundaries. The size of the model system that can be handled by MD simulation is extremely limited, corresponding to dimensions of a few nm. Accordingly, both
the phenomena treated in MD simulation and the understanding of the biological membranes would be fragmentary unless this shortcoming is overcome. The simulation box is replicated in all directions throughout the space as surrounded by its own images. If an atom leaves the simulations box through one side, it reenters through the opposite side from a neighboring box. In this way, the total number of atoms in the system is maintained constant. Frequently, a cubic or rectangular simulation box is used in lipid bilayer simulations. The lipid bilayer is positioned in the middle of the box and surrounded by water layers on both surfaces, or two monolayers are placed at the opposite sides of the box and separated by the water layer. Consequently, the simulated system represents infinite multilamellar layers by the application of the PBCs.

**Nonbonded interactions**

The calculation of the nonbonded interactions is computationally the most expensive part of MD simulations. The treatment of all the atoms present in the system in pairs is the way that the nonbonded interactions should be computed, but it would be time consuming and inefficient. In the case of the van der Waals interactions, the LJ potential is diminished to 1% of its value at $\sigma$, when the distance between two atoms reaches around $2.5\sigma$ [38]. For this reason, the calculation of the nonbonded interactions is approximated to save the computational cost by using a nonbonded cutoff. The treatment is that all the interactions between atom pairs in the system farther than the cutoff are truncated and set to zero. On the other hand, the range of electrostatic interactions is longer than that of the van der Waals interactions. A value of at least 10 Å is generally recommended for truncation of the
electrostatic interactions but this may lead to unsatisfactory results for the computation [38]. To compromise the requirement for the different cutoff ranges, a twin-range method is suggested, a shorter one for short range van der Waals interactions, and a longer one for long range electrostatic interactions [88]. The interactions present between the lower and upper cutoffs are evaluated only when the nonbonded neighbor list is updated, otherwise they remains unchanged [89].

However, the cutoff method gives rise to a problem of the discontinuity in the potential energy and the force at the cutoff distance due to the abrupt fall of the potential from its energy value to zero at the distance. To eliminate the discontinuity in the potential energy and force equations, a switching function in a polynomial form is introduced [38]:

\[
S(r) = 1 - 2 \left( \frac{r}{r_c} \right)^2 + \left( \frac{r}{r_c} \right)^4
\]  (3.13)

where \( r_c \) is the cutoff distance. Beyond the cutoff distance, the original potential function is multiplied by the switching function, whose value ranges from a value of 1 at \( r = 0 \) and a value of 0 at \( r = r_c \).

Although truncation has been widely applied to the treatment of electrostatic interactions, it is inherently a rough approximation. In the Ewald summation method, all electrostatic interactions are included in an infinite array of periodic simulation boxes by using the Ewald sum [38,90]. This avoids the problem of infinite-ranged interactions by summing over screened short-ranged interactions and eliminating the effect of screening by summing the canceling distributions in the reciprocal space [91]. A fast implementation of the Ewald
summation is the Particle Mesh Ewald (PME) method, which approximates the reciprocal space term of the Ewald sum using the Fast Fourier Transformations algorithm on a grid where charges are interpolated to the grid points [92].

**Ensembles in molecular dynamics simulations**

The choice of the statistical ensemble is one of the fundamental issues when one performs a computer simulation. A standard MD simulation produces the trajectory in the microcanonical ensemble, where the number of particles $N$, the volume $V$, and the energy $E$ of the system are constant and the temperature $T$ and the pressure $P$ are allowed to fluctuate. However, it is considered more practical to study simulation models under control of temperature and pressure, the conditions in an experimental laboratory.

Temperature of a system is directly related to the kinetic energy of the system [38,91]:

$$
\langle K \rangle = \frac{1}{2} \sum_{i=1}^{N} \frac{p_i^2}{2m_i} = \frac{3}{2} N k_B T
$$

(3.14)

where $K$ is the kinetic energy, $p_i$ is the momentum of the atom, $m_i$ is the mass, $k_B$ is the Boltzmann constant, $T$ is the kinetic temperature, and $N$ is the number of the atoms simulated in the system. Equation 3.14 implies that a way to control the temperature of the system is to scale the velocities of the atoms during the simulation [93]. The weak coupling method is simple and widely applied to control the temperature by coupling the system to a heat bath fixed at the desired temperature [24]. The coupling either removes or supplies energy to the system to maintain approximately constant temperature. The velocities are scaled at each step in order that the rate of temperature change is give by:
\[
\frac{dT}{dt} = \frac{1}{\tau_T} (T_0 - T) \tag{3.15}
\]

where \( T_0 \) is the target temperature, and \( \tau_T \) the temperature coupling constant describing the time interval between heat exchanges with the bath. If the velocities are rescaled by multiplying a factor \( \lambda \), then the change in kinetic energy upon rescaling is:

\[
\Delta K = \frac{1}{2} \left( \sum_{i=1}^{N} m_i \lambda^2 \mathbf{v}_i^2 - m_i \mathbf{v}_i^2 \right) = (\lambda^2 - 1)K = (\lambda^2 - 1)\frac{3}{2}Nk_BT \tag{3.16}
\]

Equation 3.16 can be converted to express the associated temperature change as:

\[
\Delta T = (\lambda^2 - 1)T \tag{3.17}
\]

Finally, the scaling factor for the velocities is:

\[
\lambda^2 = 1 + \frac{\delta t}{\tau_T} \left( \frac{T_0}{T} - 1 \right) \tag{3.18}
\]

which gives the relation between the scaling factor \( \lambda \), the time step \( \delta t \), and the coupling constant \( \tau_T \) whose magnitude determines the degree of the coupling between the system and the bath. If the coupling constant is large (\( \delta t \ll \tau_T \)), no rescaling takes place and the system is recovered to the microcanonical ensemble. If the coupling constant equals the time step (\( \delta t = \tau_T \)), then the simple velocity rescaling occurs as:

\[
\lambda^2 = \frac{T_0}{T} \tag{3.19}
\]
which gives the velocities multiplied by the factor \( \lambda = \frac{T_0}{\sqrt{T_c}} \) at each time step, where \( T_0 \) is the target temperature and \( T_c \) is the current temperature calculated from the kinetic energy.

The usual compromise value for \( \tau_r \) is 0.4 ps, which results in modest temperature fluctuations.

Constant pressure MD naturally involves volume changes and its implementation requires the equation of motion to describe its evolution. A pressure control method analogous to the method used for temperature control exists, coupling the system to a pressure bath [24]. The rate of change of pressure is given by:

\[
\frac{dP}{dt} = \frac{1}{\tau_p} (P_0 - P)
\]  

(3.20)

where \( P_0 \) is the pressure of the bath, and \( \tau_p \) is the pressure coupling constant. This method rescales the atom coordinates at every time step by a factor \( \chi \):

\[
\chi = 1 - \frac{\delta t}{\tau_p} (P_0 - P)
\]  

(3.21)

The new positions are given by:

\[
r_i' = \chi^\frac{1}{3} r_i
\]  

(3.22)

This method is widely used in MD simulations in conjunction with the weak coupling method for temperature control since both methods involve velocities in the right hand sides of the equations of motion, making the Verlet algorithm usable. It has been criticized, however, that
this method cannot sample any known ensembles [38].

In a new approach for performing constant pressure MD simulations, introduction of an additional degree of freedom used in the extended system method by Andersen [94] is incorporated into the pressure coupling method by a pressure bath [68]. The degree of freedom is realized by a virtual piston of mass $W$, which has a unit of (mass·length$^{-4}$). The motion of the piston is described with a Langevin equation, resulting in:

$$\frac{dr_i}{dt} = \frac{p_i}{m_i} + \frac{1}{3V} \frac{dV}{dt} r_i \quad (3.23)$$

$$\frac{dp_i}{dt} = -\nabla E(r_i) - \frac{1}{3V} \frac{dV}{dt} p_i \quad (3.24)$$

$$\frac{d^2V}{dt^2} = -\frac{1}{W} [P - P_{\text{ext}}] - \gamma \frac{dV}{dt} + R(t) \quad (3.25)$$

where $\gamma$ is the collision frequency, $P_{\text{ext}}$ is the imposed pressure, and $R(t)$ is a random force taken from a Gaussian distribution with zero mean and variance:

$$\langle R(0)R(t) \rangle = \frac{2\gamma k_B T \delta(t)}{W} \quad (3.26)$$

As driven in the Appendix in the original paper, the coupling of the piston degree of freedom to a heat bath by means of the Langevin equation results in equations of motion that produce trajectories in the isothermal-isobaric (NPT) ensemble [68].
Mean-square displacement

The mean-square displacement (MSD) is a measure of the average distance that an atom or molecule travels. It is given by:

\[ \Delta r(t)^2 = \langle |r_i(t) - r_i(0)|^2 \rangle = \frac{1}{N} \sum_{i=1}^{N} |r_i(t) - r_i(0)|^2 \]  

(3.27)

where \( |r_i(t) - r_i(0)| \) is the distance traveled by particle \( i \) over a time interval of length \( t \), and the squared magnitude of this vector is averaged over a number of such time intervals. This quantity is often averaged over all particles present in the system, summing \( i \) from 1 to \( N \) and dividing by \( N \). In a practical MD simulation, the ensemble average is replaced by time average, where \( N \) is the number of frames in trajectories.

The rate of growth of the MSD depends on the frequency of collisions that the particle suffers [75, 76]. If the particle encountered no other particles in the system, traveling ballistically, then the distance it traveled equals velocity times time and the MSD would increase quadratically with time \( t \). In a denser phase such as a liquid state, the motion of the particle is described as a random walk, for which the MSD increases only linearly with time \( t \). According to Einstein’s theory of diffusion, the limiting slope of the MSD is related to the self-diffusion coefficient \( D \) when time intervals are sufficiently long enough for the MSD to be in the linear regime as following [38]:

\[ \lim_{t \to \infty} \frac{d}{dt} \langle |r_i(t) - r_i(0)|^2 \rangle = 2dD \]  

(3.28)

where \( d \) is 2 for 2-dimensional diffusion and 3 for 3-dimensional diffusion.
Radial distribution function

The radial distribution function (RDF) is a useful way to quantify the motion of particles in the system in an average sense. If particles are uncorrelated, then the expected number of particles at \( r \) is simply the average number of particles per unit volume times the volume of the shell of radius \( r \) and thickness \( dr \):

\[
g^{id}(r) = \rho^{id}(r) \times V_{shell}(r) = \frac{N}{V} \times 4\pi r^2 dr
\]  

(3.29)

where \( \rho^{id}(r) \) is the number density in the ideal system. To quantify preferential correlations, it is necessary to normalize the observed number of particles at \( r \) by the number that would be observed if the particles are uncorrelated. The ratio is the radial distribution function, commonly referred as:

\[
g(r) = \frac{\rho(r) dr}{\rho^{id}(r) dr}
\]  

(3.30)

For pairwise additive potentials, the knowledge of the RDF provides sufficient information that can calculate thermodynamic properties, particularly energy and pressure [38]. The RDF can be measured experimentally, using X-ray diffraction or neutron-scattering techniques [38].
4. MOLECULAR DYNAMICS SIMULATIONS OF SMALL MOLECULE DIFFUSION THROUGH BIOLOGICAL MEMBRANES

Components of microscopic model systems

Two types of microscopic model systems were constructed for MD simulations of biological membranes. The first model is a fully hydrated lipid bilayer, representing simple biological membranes. The hydrated lipid bilayer model is also used to validate the previous simulations of the transport of small molecules through DPPC bilayers [10,22]. The second model is a gas channel protein embedded in the hydrated lipid bilayer as a more complex form of biological membranes. The two models allow comparison of the rate of the diffusion of ammonia and carbon dioxide through the different types of biological membranes.

Lipid molecules are the major constituents of the biological membrane. The lipid molecules consist of headgroup molecules and two fatty acid chains. The form of the lipid molecules varies with respect to the kind of headgroup molecules and the length and degree of saturation of each fatty acid chain. Typical headgroup molecules are choline and ethanolamine. The length of the fatty acid chain is often in the range from 14 to 20 carbons.

For use in models and studies on cell membranes, phosphatidylcholine (PC) lipids have been considered as a good model for biological membranes. In the current simulations, palmitoyloleoylphosphatidylcholine (POPC) molecules are used to represent the cell membrane in a bilayer configuration. As shown in the previous chapter, DMPC and DPPC
lipid molecules have been employed to represent a realistic model lipid bilayer in a number of computer simulations [10,22,16,17,71,74,77,82,83,85]. However, the existence of double bonds in the specific positions of the fatty acid chains of the phospholipids is known to be essential for the form and functionality of biological membranes [95]. For example, the degree of unsaturation is an important factor for the shape and the function of hemolysis and permeation of the human erythrocyte [96]. In nature, phospholipids with two asymmetric fatty acid chains are the most typical in the form of mono-\textit{cis} or poly-\textit{cis}-unsaturated PCs [97]. The abundance of POPC among the mono-\textit{cis}-unsaturated PCs [98] motivated the choice of POPC as a basal component of the model for a lipid bilayer membrane.

The crystallographic structure of the AmtB gas channel protein [30] was downloaded from the protein data bank (PDB) online [99], access code 1U7G, and embedded in the lipid bilayer. The crystallographic structure of the transport protein was experimentally obtained from the bacterium \textit{E. coli} by X-ray diffraction at the resolution of 1.35 Å, the highest resolution structure of any membrane protein to date. The 1U7G protein contains three ammonia molecules as heterogroup molecules around the two histidine residues (\textit{His}^{168} and \textit{His}^{318}). The point group of the protein structure is P6\textsubscript{3} with the unit cell dimensions \(a = 95.64 \text{ Å}, \ b = 95.64 \text{ Å}, \) and \(c = 94.62 \text{ Å}\) and angles \(\alpha = 90^\circ, \ \beta = 90^\circ, \) and \(\gamma = 120^\circ.\) The total number of residues and atoms are 385 and 3,249, respectively. In addition to 1U7G, Zheng et al. isolated another crystallographic structure of AmtB at the resolution of 1.80 Å [31]. The crystallographic structure can be accessed on the PDB online with the access code 1XQF. The two AmtB proteins are slightly different in the dimensions of the unit cell and the number of residues and atoms. However, the calculated path for a solute molecules and the available free volume in the protein channel and around the channel vestibules are significantly
different from each other, as presented in Figure 4.3 and 4.4. The calculation of the path and the volume of the AmtB proteins are performed with the HOLE algorithm [100] that allows the analysis and visualization of the molecular structure of channels with a graphic interface. The HOLE algorithm calculates the van der Waals radius of each atom given in a coordinate file along the plane normal to the channel vector in order to find the largest available sphere that can be accommodated without overlap with the van der Waals surface of any atom. In the MD simulations of the membrane channels, 1U7G is selected for the model channel protein since 1U7G contains the most detailed molecular structures of the channel with ammonia molecules. Additionally, the calculated path for transport of solute molecules passes through the protein from the top and the bottom of the channel such as in a normal pore, while the path for 1XQF is shown to go through the side of the protein channel.

Bulk water layers used to be located adjacent to the headgroup region of the lipid bilayers as a universal component of the membrane. Among simple water models using between three and five interactions sites and a rigid water geometry, TIP3P [101] and SPC [102] models use a total of three sites for the electrostatic interactions and possess a slight difference between their geometries, hydrogen charges, and LJ parameters [38]. The choice of an appropriate water model for MD simulations of the biological membrane simulation has to be limited within the simple rigid water models due to the computational cost for the calculation of the intensive electrostatic calculations [103].

The solute molecules allowed to diffuse through the two membrane models are ammonia and carbon dioxide. A set of the CHARMM force field parameters of ammonia for the MD simulations are taken from the OPLS-AA (Optimized Parameters for Lipid Simulations – All Atom) force field parameters [104]. The most current version of the
CHARMm force field (Release c31b1) contained the set of parameters and residue topology for carbon dioxide.

**Construction of model systems**

The construction of the microscopic model systems was performed using VMD (Visual Molecular Dynamics) [105] as a primary tool and its script and plug-in libraries, which mostly require a residue topology file as a part of the force field parameters. VMD is a molecular graphics program developed by the Theoretical Biophysics group at the University of Illinois and the Beckman Institute, specially designed for the visualization and analysis of large biomolecular systems. The procedures for construction of the two model systems are different. For the hydrated lipid bilayer, the construction of the lipid bilayer with POPC molecules is followed by adding water layers on each side of the bilayer. Ammonia and carbon dioxide molecules are added at designated positions. For the membrane-protein complex, the AmtB protein is embedded in the middle of the POPC lipid bilayer along with the z – axial direction to construct the membrane channel model. Bulk water layers are added on both sides of the membrane-protein complex. Ammonia and carbon dioxide is added at which the two histidine residues are within the AmtB protein.

By using the VMD membrane plug-in, 128 POPC molecules were randomly generated in a rectangular matrix of the required size from previously equilibrated membrane square patches with solvated lipid headgroup molecules. The fatty acid chains of the lipid were almost fully extended to allow unproblematic insertion of the protein into the lipid bilayer. The spacing between the two monolayers and the lattice period of the bilayer were set to fit to
the parameters available from experiments [106]. For proper hydration of the lipid headgroup molecules, the water molecules from the pre-built membrane patches were removed, and then the dehydrated lipid bilayer was resolvated by VMD solvate plug-in. The solvate plug-in also exploits the pre-equilibrated water box and shell-by-shell hydration around an object. The final structure of the fully hydrated POPC bilayer consisted of randomly generated 128 POPC and 3,528 TIP3P water molecules that were mostly located around the lipid headgroup molecules. The number of water molecules per POPC headgroup is believed to be approximately 27 to sufficiently hydrate a POPC molecule [98]. The initial dimension of the simulation box containing the lipids and the solutes is $72 \times 72 \times 64 \, \text{Å}^3$.

The initial position of ammonia and carbon dioxide into the lipid bilayer was located at two different regions. The first is near the interface between the water layer and the lipid headgroup molecules and the second is at the interior of the lipid bilayer. The $z$ coordinates of the inserted solute molecules were $z = 21 \, \text{Å}$ and $z = 1 \, \text{Å}$ in the region of the interface and the interior, respectively. The two different initial positions of ammonia are shown in Figure 4.1 (a) and (b). For $x$- and $y$-coordinates, the molecules were placed at the center of the quarterly divided part in the top monolayer. The determination of the interfacial position of the solute molecules were based on the coordinates of phosphorous and nitrogen atoms in the POPC headgroup. The variation of 20 Å on the initial positions between the solute molecules was motivated by the expected displacement of the solute molecules within the AmtB channel protein. Each hydrated POPC bilayer contained four solute molecules with different initial positions for each run. Consequently, eight different positions were sampled for both ammonia and carbon dioxide.

The membrane-protein complex was constructed by inserting the AmtB channel
protein into the lipid bilayer using VMD and a Tcl (Tool command language) script available at the membrane plug-in website [107]. The Tcl script is written for merging the protein and the lipid bilayer and removing lipids that overlap with the protein groups, requiring proper orientation and alignment of the lipid bilayer and the protein before the script is executed.

However, the AmtB coordinate file (1U7G.pdb) includes a group of residues whose force field parameters are not available in the CHARMM 31. Since the imidazole side chain of histidine exists in two tautomeric forms with a hydrogen atom moving between the two nitrogen atoms, two different neutral histidine residues are available in the CHARMM 31 residue topology file. As a result, the two His\(^{168}\) and His\(^{318}\) residues were named as HSD and HSE, respectively, in the newly generated coordinate and structure files of the protein. In the topology file, HSD means the δ nitrogen of histidine is protonated, while HSE means the ε nitrogen is protonated.

The rest of the histidine residues present in the protein structure and coordinate files were named as HSE. The position of ammonia molecules was already determined from the downloaded coordinate file (1U7G.pdb) to be around the two histidine residues at \(z = -5.41\ \text{Å}, -1.98\ \text{Å},\) and \(1.06\ \text{Å}\). Carbon dioxide molecules were included at those \(z\) coordinates by replacing the nitrogen atoms of ammonia with the carbon atoms of carbon dioxide. The initial positions of ammonia are shown in Figure 4.2. Finally, the starting structure for MD simulation is the assembly of the AmtB channel protein, the lipid bilayer with 72 POPC molecules, and 3,957 TIP3P water molecules in \(72 \times 72 \times 72\ \text{Å}^3\).

**Preparation for molecular dynamics simulations**

A series of MD simulations were conducted to study the diffusion of ammonia and
carbon dioxide through the POPC bilayer and the AmtB channel protein with NAMD (Not Another Molecular Dynamics) [108] developed by the Theoretical Biophysics group at the University of Illinois and the Beckman Institute. NAMD is a parallel, object-oriented molecular dynamics code designed for a high-performance computing (HPC) environment for large biomolecular simulations. NAMD is compatible with such file formats used by AMBER and CHARMm and freely distributed with the source code for its flexibility and functionality. All NAMD runs were executed on the Pentium™ IV cluster at the Ohio Supercomputer Center (OSC) using 16 processors throughout the simulations.

To run NAMD requires a coordinate file (.pdb), a structure file (.psf), a parameter file (.prm), a residue topology file (.rtf), and a NAMD configuration file of the system. The coordinate file possesses information such as atom name, residue name, rectangular coordinate, occupancy, and temperature factor (B_j) factor. The structure file contains all of the molecule-specific information in five main sections of interest, atoms, bonds, angles, dihedrals, and impropers, necessary to apply a particular force field to a molecular system. The parameter file has a number of sets of force field parameters for the limited number of molecules of such as lipids, amino acids, ions, and nucleic acids. The topology file includes atom mass and internal coordinates, partial charges, and multitude of bonds of specific residues. The configuration file includes a number of simulation protocols to execute MD under designated conditions. Both coordinate and structure files of the POPC bilayer were generated during the construction with the VMD membrane plug-in. For the solute molecules, the coordinates of the nitrogen of ammonia and the carbon of carbon dioxide were manually inserted into the lipid bilayer coordinate files. Then, using VMD psfgen plug-in allowed having the structure file containing the molecules of lipid, solute, and water. Combine.tcl script [107]
automatically generated the coordinate and structure files of the membrane-protein complex with respect to the changes by the embedment of the transport protein into the POPC bilayer. In this work, the CHARMm 31 force field parameters and the OPLS-AA parameters for ammonia were employed for the MD simulations although the parameter and residue topology files of the CHARMm 27 (Release c27b1) are originally provided with NAMD 2.5 (Sep. 2003) and VMD 1.8.3 (Apr. 2005).

The estimated diffusion coefficients of ammonia and carbon dioxide and the dimension of the probable diffusion of the solute molecules predict the length of simulation time. An experimentally measured diffusion coefficient for ammonia has not yet been reported in biological membranes [22]. In calculation of the rate of carbon dioxide uptake by red blood cells, the diffusion coefficient in the exchange of oxygen and carbon monoxide is $6.5 \times 10^{-6}$ cm$^2$/s [57,109]. In ammonia transport through the AmtB channel protein, the diffusion coefficient $D$ was estimated to be $3.7 \times 10^{-6}$ cm$^2$/s by Fick’s first law of diffusion:

$$J = -D \frac{dc}{dx}$$  \hspace{1cm} (4.1)

where $J$ is the flux of ammonia, ranging from 10 to 10,000 molecules per second per channel [31], $dc$ the concentration difference of 8 μM in the channel for the protonation of ammonia [110], and $dx$ the channel length 20 Å. By using the Einstein relation [27]:

$$d^2 = 6Dt$$  \hspace{1cm} (4.2)

the time required the solute molecules diffuse through the 20 Å-long channel would be 1.8 ns and 1.0 ns for ammonia and carbon dioxide, respectively.
Equilibration and production phases

Equilibration phase is usually divided into minimization and equilibration. Minimization involves searching a nearby local energy minimum of the microscopic model systems since the initial coordinates of the systems contain unfavorable contacts that cause anomalous energies and forces. After minimization, the energy of the system is allowed to escape the local minimum with low energy barriers to reach thermodynamically well-distributed states throughout the systems. Minimization and equilibration of the hydrated lipid bilayer and the membrane-protein complex systems were performed for 500 ps. Two simulations for equilibration for each solute molecule in the hydrated lipid bilayer and the membrane-protein complex systems were conducted, resulting in a total of eight simulations. The MD time step was set to 2 fs with SHAKE algorithms [111] by which all bonds involving hydrogen atoms were constrained throughout the simulations. All pair interactions were calculated up to 10 Å, but between 10 Å and 12 Å, only electrostatic interactions were taken into account using a list that was updated every 20 time steps. The switching function was effective from 8 Å to 12 Å. After 100 ps minimization and 50 ps heating in series, Langevin dynamics was employed to equilibrate the hydrated lipid bilayer with relaxing the restraints on carbon alpha atoms after 200 ps. In the membrane-protein complex system, Langevin dynamics was performed for 50 ps to fill the gap between the channel protein and the lipid bilayer to prevent water molecules from penetrating into the gap before 50 ps minimization which is followed by 50 ps heating. For the remaining 350 ps, Langevin dynamics equilibrate the membrane-protein complex system with restraints on carbon alpha atoms for 50 ps and
without restraints for 300 ps. The Langevin temperature and the piston target pressure were set to 310 K and 1.013 bar, respectively, throughout the equilibration phases.

There are several methods to determine if systems become stable after equilibration. For the lipid bilayers, density and area per lipid are the properties that should be stabilized during equilibration [9]. If the number of atoms present in the system remains constant, volume of the system is linearly proportional to the density of it. Figure 4.5 shows that the volume of the hydrated lipid bilayer is stabilized during the simulations for both systems containing ammonia and carbon dioxide. In addition, Figure 4.6 shows the convergence of the total energy during equilibration phases for both of the systems. The conformational variations of the protein within the membrane-protein complex was examined by the RMSD after equilibration with respect to the initial structure as a reference [9,17]. Figure 4.5 shows that the RMSD of the solutes in the AmtB channel protein fall in the range from 2 Å to 3 Å deviation, indicating no significant fluctuation of the protein structure in the POPC bilayer during the equilibration phase.

For the final production phase, 1 ns NPT dynamics were performed for the hydrated lipid bilayer systems. The switching function was turned on at a distance of 6.5 Å for nonbonded interactions. The cutoff and the pairlist distance were set to 8.5 Å and 10.5 Å, respectively. The initial structures for NPT dynamics were shrunken from $72 \times 72 \times 64 \, \text{Å}^3$ to approximately $66 \times 66 \times 66 \, \text{Å}^3$ for the systems including ammonia molecules and $67 \times 67 \times 64 \, \text{Å}^3$ for those of carbon dioxide. The Langevin temperature and the piston target pressure were set to 350 K and 1.013 bar, respectively, whose conditions were set for the study of permeation of ammonia through the DPPC bilayer [22]. The Langevin damping coefficient was set to 5 ps$^{-1}$, which corresponds to the coupling constant 0.1 ps in the permeation study [22], by the
following relationship:

\[ \tau_f = \frac{1}{2\gamma} \]  

(4.3)

where \( \gamma \) is the damping coefficient in a Langevin equation [24]:

\[ m \frac{d^2\mathbf{r}}{dt^2} = \mathbf{F}_f - m \gamma \frac{d\mathbf{r}}{dt} + \mathbf{R}(t) \]  

(4.4)

where \( \mathbf{R}(t) \) is a Gaussian stochastic variable defined as Equation 3.26. The Langevin piston decay time was set to 0.5 ps by the relationship that \( \tau_p \) is approximately equal to the decay time of the piston mass [68]. The actual time duration of NPT dynamics was set to 1.1 ns to discard the first 100 ps for the temperature change from 310 K in the equilibration phase to 350 K in the production phase. For the membrane-protein complex systems, 2.5 ns NVT dynamics were conducted. The initial structures for NVT dynamics were reduced to approximately 63 \( \times \) 63 \( \times \) 72 Å\(^3\). The parameters for the nonbonded interactions remained unchanged, used in the hydrated lipid bilayer simulations. The system temperature was set to 310 K with the Langevin damping coefficient 5 ps\(^{-1}\).

**Results**

The behavior of the molecules in each system and the final simulation structures are described in this section. The size of the simulation box changed to 67 \( \times \) 68 \( \times \) 67 Å\(^3\) with minor variations in the magnitude. The shape of the box remains to be orthorhombic. For
the hydrated lipid bilayers, water molecules diffuse only up to the lipid headgroup region, reaching \( z = 9 \text{ Å} \) to \( 11 \text{ Å} \) for the top monolayer and \( z = -9 \text{ Å} \) to \( -12 \text{ Å} \) for the bottom monolayer. The POPC molecules are significantly randomized and several fatty acid chains of them protrude out of the simulation box. Generally, no preference for the lateral movement of the solute molecules is observed. In the case of ammonia molecules placed at the headgroup region, two out of the four sampled ammonia molecules stay near the headgroup region intermittently for 30 to 50 ps. Otherwise, the ammonia molecules diffuse out of the lipid bilayer and wander around the bulk water phase. In the case of ammonia molecules placed at the center of the lipid bilayer, all the ammonia molecules reach the interface between the water layer and the lipid bilayer after 360 ps. In the case of carbon dioxide placed at the headgroup region, three out of four carbon dioxide molecules move into the interior of the lipid bilayer after 470 ps. Those three molecules are not able to reach the bottom monolayer of the lipid bilayer. The carbon dioxide molecules diffusing into the interior of the lipid bilayer moved less frequently, since the fatty acid chains of the lipid hinder the movement of molecules inside the bilayer. This is held true for ammonia molecules that diffused out to the bulk water phase and traveled faster than carbon dioxide trapped within the lipid bilayer. In the case of carbon dioxide placed at the center of the bilayer, one carbon dioxide molecule reached the bottom monolayer of the lipid bilayer, but it was not allowed to pass through the interface between the lipids and the bulk water phase.

For the membrane-protein complex, significant changes of the free volume within and around the protein are accompanied after the simulations as presented in Figure 4.8 and 4.9. Water molecules are able to arrive at the position \( z = -7 \text{ Å} \) and \( 5 \text{ Å} \), while the \( \delta \) nitrogen molecules of the two histidine residues are located at approximately \( z = -5 \text{ Å} \) and \( 2 \text{ Å} \).
Ammonia molecules are lined such in a single file and would stay close to the two histidine residues. Carbon dioxide molecules show no preferential association with the two conserved histidine residues. After 1,100 ps, one of the three carbon dioxide molecules diffused out to the interface between the lipids and the protein. At 900 and 1,060 ps, one water molecule at a time penetrated into the vicinity of the His$^{318}$ and diffused back to the bottom vestibule of the protein at 2,260 ps. The CPU times to complete equilibration and minimization for each system are summarized in Table 4.1. The CPU times to complete final production phases for each system are summarized in Table 4.2.
Figure 4.1  The initial positions of ammonia molecules in the hydrated POPC lipid bilayer system, visualized by VMD. The water molecules are represented by the point method, the lipid molecules are represented by the line method, and the ammonia molecules are represented by the VDW method. (a) The initial position at the interface region, z = 21 Å; (b) the initial position at the interior region, z = 1 Å.
Figure 4.2  The initial positions of ammonia molecules in the membrane-protein complex system, visualized by VMD.  The AmtB protein is represented by the *ribbon* method.  The ammonia molecules are at $z = -5.41$ Å, -1.98 Å, and 1.06 Å.
Figure 4.3  The probable path of movement of a solute and accessible area of the AmtB protein (1U7G) with respect to a radius of a molecule are calculated by the HOLE program and visualized by VMD.  The protein is drawn by the *ribbon* method and the histidine residues by the *bond* method.  The yellow line represents the probable path of movement of a solute within the transport protein.  The dotted surface is categorized according to the accessibility of molecules.  The blue color denotes open pores accessible by molecules whose radius is larger than 2.3 Å.  The region colored green is accessible by molecules whose radius between 1.15 Å and 2.3 Å.

(a) View from normal to the z-axis of the protein; (b) view from the top of the protein
Figure 4.4 The probable path of movement of a solute and accessible area of the AmtB protein (1XQF) with respect to a radius of a molecule are calculated by the HOLE program and visualized by VMD. The region colored red is inaccessible to molecules whose radius is less than 1.15 Å.

(a) View from normal to the z-axis of the protein; (b) view from the top of the protein
Figure 4.5 The volume in unit of Å$^3$ of the hydrated lipid bilayers during the equilibration for 500 ps.

(a) The lipid bilayer containing ammonia; (b) the lipid bilayer containing carbon dioxide
Figure 4.6 The energy in unit of kcal/mol of the hydrated lipid bilayers during the equilibration for 500 ps.

(a) The lipid bilayer containing ammonia; (b) the lipid bilayer containing carbon dioxide

![Figure 4.6](image)

Figure 4.7 The RMSDs in unit of Å of the AmtB protein with ammonia and carbon dioxide during the equilibration for 500 ps.

![Figure 4.7](image)
Figure 4.8  The final free volume of the system containing ammonia as solutes after 2.5 ns NVT dynamics. The ammonia molecules are drawn by the $VDW$ method. Nitrogen and hydrogen atoms are colored in blue and white, respectively.  (a) View from normal to the z-axis of the protein; (b) view from the top of the protein.
Figure 4.9  The final free volume of the system containing carbon dioxide as solutes after 2.5 ns NVT dynamics. The carbon dioxide molecules are drawn by the $VDW$ method. Oxygen and carbon atoms are colored in red and cyan, respectively. (a) View from normal to the $z$-axis of the protein; (b) view from the top of the protein.
Table 4.1  The CPU times to complete minimization and equilibration for 500 ps for each system.

<table>
<thead>
<tr>
<th>System</th>
<th>CPU time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid bilayer with ammonia at interface(^a)</td>
<td>37,765</td>
</tr>
<tr>
<td>Lipid bilayer with ammonia at interior(^b)</td>
<td>26,151</td>
</tr>
<tr>
<td>Lipid bilayer with carbon dioxide at interface</td>
<td>32,019</td>
</tr>
<tr>
<td>Lipid bilayer with carbon dioxide at interior</td>
<td>31,844</td>
</tr>
<tr>
<td>Membrane-protein complex with ammonia</td>
<td>32,019</td>
</tr>
<tr>
<td>Membrane-protein complex with carbon dioxide</td>
<td>31,844</td>
</tr>
</tbody>
</table>

\(^a\): The interface refers to the initial position of the solute at \(z = 21 \text{ Å}\).

\(^b\): The interior refers to the initial position of the solute at \(z = 1 \text{ Å}\).

\(^c\): Performed with 16 processors.
Table 4.2 The CPU times to complete 1.0 ns NPT dynamics for the lipid bilayer system and 2.5 ns NVT dynamics for the membrane-protein complex system.

<table>
<thead>
<tr>
<th>System</th>
<th>CPU time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid bilayer with ammonia at interface</td>
<td>42,437</td>
</tr>
<tr>
<td>Lipid bilayer with ammonia at interior</td>
<td>35,478</td>
</tr>
<tr>
<td>Lipid bilayer with carbon dioxide at interface</td>
<td>37,609</td>
</tr>
<tr>
<td>Lipid bilayer with carbon dioxide at interior</td>
<td>41,253</td>
</tr>
<tr>
<td>Membrane-protein complex with ammonia</td>
<td>62,919</td>
</tr>
<tr>
<td>Membrane-protein complex with carbon dioxide</td>
<td>68,822</td>
</tr>
</tbody>
</table>
5. FINAL ANALYSIS

Analysis of the MD simulations is concentrated on the rate of the transport of ammonia and carbon dioxide through the hydrated lipid bilayers and the gas channel protein embedded in the lipid bilayers. Another emphasis of the analysis is made on the evaluation of preferential association between the $\delta$ and $\epsilon$ nitrogen atoms and the solutes allowed to pass through the transport protein. The gOpenMol [112,113] software was used to analyze trajectories for calculations of the MSD and the RDF.

Diffusion coefficients

The diffusion coefficients of the solutes are calculated by the relationship of Einstein’s theory of diffusion with the MSD in Equations 3.27 and 3.28. The validity of the relationship is tested by the following equation indicating diffusion mechanism with respect to the power $n$. Where $n$ is 0 for anomalous diffusion, 1 for Einstein diffusion, and 2 for ballistic motion of particles [76]:

$$\log(\text{MSD}) \propto n \cdot \log(t)$$  \hspace{1cm} (5.1)

If $n = 1$, then the gradient of a MSD curve with simulation time yields the right hand side of Equation 3.28. In practice, diffusion coefficients are calculated by taking the linear fraction
of the MSD curve while isolating it from the unsteady increase of the MSD.

In the diffusion of ammonia through the POPC bilayer, the diffusion coefficient of ammonia is calculated to be in the order of $10^{-5}$ cm$^2$/s, showing good agreement in the order of magnitude of the local diffusion coefficients of ammonia through the headgroup region of the DPPC bilayer [10, 22]. In addition to quantitative comparison of the rate of diffusion, the dynamic behavior of the system is also agreeable to the prediction. By yielding the slopes in Figure 5.1 (a), the ammonia molecules initially placed at the headgroup region into and out of the lipid bilayer are comparable to random walk motions. However, the situation for the ammonia molecules whose initial position was at the center of lipid bilayer is significantly different, resulting in ballistic motion of the ammonia molecules within the lipid bilayer. This is supported by showing that the gradient of the “interior” curve in Figure 5.1 (a) is approximately two before 400 ps, which is the onset of the random walk in the lipid bilayer for 120 ps. In terms of the structural change of the lipid bilayer, this type of motion is closely related to the increase of the free volume at the interior region of the lipid bilayer by setting the temperature of the NPT simulations at 350 K. After approximately 360 ps when all the ammonia molecules have reached the headgroup region, the ammonia molecules stop showing ballistic motions and fall in the region of the random walk motions. Beyond 520 ps, the movement of the ammonia molecules is limited within the interfacial region between the bulk water phase and the headgroup region.

In carbon dioxide diffusion, the general trend of the rate of diffusion is slower than the rate of the diffusion of ammonia as shown in Table 5.2. A theoretical expression for the diffusion coefficient for gas pairs of nonpolar and nonreacting molecules is presented using the LJ potential [115]:

$$D = \frac{kT}{6\pi\eta R}$$
\[
D_{AB} = \frac{0.001858T^{3/2} \left( \frac{1}{M_A} + \frac{1}{M_B} \right)^{1/2}}{P\sigma_{AB}^2 \Omega_D}
\] (5.2)

where \( D_{AB} \) is the mass diffusivity of \( A \) through \( B \) in cm\(^2\)/s, \( T \) is the absolute temperature in K, \( M_A \) and \( M_B \) are the molecular weights of \( A \) and \( B \), respectively, \( P \) is the absolute pressure in atm, \( \sigma_{AB} \) is the collision diameter of a LJ parameter in Å, and \( \Omega_D \) is the collision integral for molecular diffusion, a dimensionless function of the temperature and of the intermolecular potential-field for one molecule of \( A \) and one molecule of \( B \). Since the diffusion coefficient of gas molecules is inversely proportional to the square root of the collision diameters, the diffusion coefficient of carbon dioxide should be smaller than that of ammonia incorporating Equation 5.2 and the collision diameters in Table 5.3. The change of the dynamic behavior of the carbon dioxide is accompanied albeit is seems to be less significant than the case of ammonia diffusion as shown in Figure 5.1 (b). The rate of the diffusion of carbon dioxide from the headgroup region was retarded after 470 ps since three carbon dioxide molecules reached the region of the highest density of the lipid bilayer, where molecular motions are significantly hindered. On the other hand, the rate of diffusion of carbon dioxide initially placed at the interior region of the lipid bilayer is in relatively unsteady increase corresponding to the random distribution of free volume within the lipid bilayer, while the equilibrium region is isolated from 310 to 526 ps.

The displacement of ammonia and carbon dioxide was obtained in two different ways as presented in Table 5.2. The overall displacement of the solutes is the square root of the MSD calculated with the trajectories of the solutes throughout the overall simulation time.
However, diffusion coefficient is calculated by the Einstein relation during time intervals regarded as purely or at least roughly in the motion of the random walk. Consequently, the effective displacement of the solutes is the displacement over specified time intervals such as in Table 5.1.

The displacement of the solutes is influenced by their size and the initial position. The displacement of ammonia molecules initially placed at the headgroup region is more than the average distances between the lipid headgroup molecules in each monolayer (approximately 40 Å). The lateral movement of ammonia near the headgroup region is more pronounced than the longitudinal movement due to the high density of the headgroup region. The displacement of carbon dioxide placed at the interior region of the lipid bilayer is less than half the longitudinal dimension of it. This is attributed to the effect from the size of carbon dioxide resulting in the retarded passage of carbon dioxide molecules out of the lipid bilayer. However, the displacement of the ammonia molecules whose initial position is the interior region of the lipid bilayer is longer than that of carbon dioxide molecules since the ammonia molecules effectively moves out of the lipid bilayer after 360 ps. Considering the result from the displacement of the solutes initially placed at the interior region of the lipid bilayer, the size effect contributes to the MSD of the solutes and thus leads to the change of the diffusion coefficients of the solutes. However, switching the lipid molecules in the lipid bilayer from DPPC to POPC has no apparent changes on the diffusion coefficients of the solutes.

Finally, the diffusion coefficients of ammonia and carbon dioxide through the hydrated POPC bilayer from the MD simulations are in good agreement with the local diffusion coefficients in the DPPC bilayer, considering the order of magnitude of the diffusion coefficients and the effect of the size. The diffusion coefficients obtained from the MD
simulations only consider the diffusion mechanism with purely or approximately random walk motions of the solutes, while the study of permeation of ammonia through the DPPC bilayer evaluates the local diffusion coefficients of lateral movement of ammonia. For evaluation of the diffusion coefficients, the solutes were allowed to diffuse in any directions in the lipid bilayer microscopic model systems while varying the initial positions of the solutes in the lipid bilayer.

The diffusion coefficients of ammonia and carbon dioxide in the membrane-protein complex were calculated by applying the identical methods employed for the hydrated lipid bilayer systems. The diffusion coefficients shown in Table 5.5 are the order of $10^{-8}$ cm$^2$/s, indicating significantly lowered rate of diffusion of ammonia and carbon dioxide through the AmtB channel protein. The reason why the rate of diffusion through the AmtB channel is lower than that through the hydrated lipid bilayer is attributed to driving force and different structure in the two different systems. In the lipid bilayer system, ammonia and carbon dioxide molecules have their own kinetic energy due to temperature of the system maintained at 350 K. The ammonia and carbon dioxide molecules move freely along the available free volume in the bilayer. In the membrane-complex system, the ammonia and carbon dioxide molecules also have kinetic energy corresponding to temperature 310 K. However, the energy barrier for the solute molecules to diffuse through the protein channel is higher than the energy furnished with system temperature at 310 K. In addition, the displacement of the solutes in the protein channel provides evidence for the significant reduction of the rate of the diffusion of the solutes. From the final structure of the AmtB channel protein shown in Figure 4.8 and 4.9, the displacement of ammonia and carbon dioxide is confined within the free volume around the two conserved histidine residues. In the case of the diffusion of
carbon dioxide, however, one of the carbon dioxide molecules diffused out to the interface between the POPC lipids and the protein. In Figure 4.9, the increase of the accessible region by molecules is located in the interface rather than in the vestibules of the channel. This explains the reason why the rate of diffusion of carbon dioxide is faster with relatively larger displacement of carbon dioxide than that of ammonia with relatively larger displacement of carbon dioxide. However, the remaining two carbon dioxide molecules reside in the free volume generated. The overall displacement of the solutes presented in Table 5.5 is only comparable to the size of the collision diameters of the solutes or slightly larger, resulting in significantly smaller diffusion coefficient. The following section will discuss the structure and the association of the solutes within the channel by obtaining the RDF between the solutes and the nitrogen molecules in histidine residues.

Slow rate of diffusion of ammonia or ammonia cation through the AmtB channel protein has been believed to be related to structural fluctuation of the channel entry and its hydrophobic environment into the channel around the bulky side chains [31]. According to the system behavior discussed in Chapter 4, the dynamic movement and the hydrophobicity of the channel entry show no contribution to the control of conduction of the solutes and water. Penetration of water molecules from the bulk water phase is also not observed as in the case of NAMD simulation by Khademi et al. [30], however, water molecules reached the vicinity of the two conserved histidine residues up to $z = -7 \, \text{Å}$ and $5 \, \text{Å}$, from the bottom and the top of the channel, respectively.

*Radial distribution functions*
Structural information within the channel is provided by the RDFs between the solutes and the δ and ε nitrogen atoms in the histidine residues. In Figure 5.3 and 5.4, the RDFs are obtained to reveal the association between the solutes and the nitrogen atoms in three different ways: between the solutes and the δ and ε nitrogen atoms in all histidine residues in the AmtB channel protein, between the solutes and the δ and ε nitrogen atoms in His\textsuperscript{318}, and between the solutes and the δ and ε nitrogen atoms in His\textsuperscript{168}, where His\textsuperscript{168} and His\textsuperscript{318} are the two conserved histidine residues in the AmtB channel protein.

In the case of the diffusion of ammonia, the first sharp peaks of the RDFs between ammonia and the δ and ε nitrogen atoms in all histidine residues in the AmtB channel protein in Figure 5.3 (a) are at \(r = 3.6\) Å and 5.4 Å with approximately identical heights of 64. The heights of the following peaks are reduced to approximately 5.5 at \(r\) fluctuating around 20 Å. The first sharp peaks indicate a strong association between ammonia and the histidine residues, leading to good agreement on the distance between ammonia and the histidine residues from the crystallographic structures [30]. The distances range from 2.6 Å to 3.4 Å. The successive peaks are present at \(r\) approximately 20 Å, providing information of the positions of the rest of histidine residues in the AmtB channel protein. This dimension corresponds to the length of the channel size for diffusion inside the hydrophobic channel. The RDFs between ammonia and each histidine residue in Figures 5.3 (b) and (c) show considerably increased height providing evidence for strong association between ammonia and the histidine residues. The RDF between ammonia and the ε nitrogen of His\textsuperscript{168} has two sharp peaks at \(r = 2.2\) Å and 3.2 Å. Otherwise in Figures 5.3 (b) and (c), the general shape of the peaks are one broad peak at \(r\) from 3.8 Å to 5.4 Å in each case, suggesting a close existence of ammonia molecules around the histidine residues during most simulation time. Figure 4.8 can demonstrate the
structure of all three ammonia molecules around the histidine residues.

The preferential association of carbon dioxide to the histidine residues is less strong than that of ammonia shown in Figure 5.4. However, the number of peaks is at least three in all cases. These results lead to a conclusion that the association between carbon dioxide and the $\delta$ and $\varepsilon$ nitrogen atoms in the histidine residues is less strong and that the association among carbon dioxide is weaker than the case of ammonia diffusion through the AmtB channel protein. The comparison of the strength of association in the diffusion of ammonia and carbon dioxide supports a conclusion that the substance for the AmtB channel protein is ammonia and the substrate for the Rh-protein is carbon dioxide [35]. Since the condition for diffusion through the AmtB channel protein is maintained constant throughout the MD simulation, the difference of the degree of association can only be attributed to the difference of the solutes. Qualitatively, the preferential association between the His$^{318}$ and the solutes is approximately 10 times, while the preferential association between His$^{168}$ and the solutes is approximately 30 times. The heights of the first peak in Figure 5.4 (a) are approximately 40 and 6.5 of the RDFs between carbon dioxide and the $\delta$ and $\varepsilon$ nitrogen atoms in all histidine residues in the AmtB channel protein. The RDFs between carbon dioxide and the two conserved histidine residues in Figure 5.4 (b) and (c) are completely diminished beyond approximately 30 Å, nearly half the dimension of the simulation box and more than the hydrophobic channel length for diffusion. The last peaks in Figure 5.4 (b) and (c) are attributed to the one carbon dioxide molecule that traveled to the interface normal to the $z$-axis between the protein and the lipid bilayer.
Figure 5.1  Log(MSD) vs. log(time) of (a) ammonia; (b) carbon dioxide during 1 ns NPT dynamics. The interface and the interior refer to the initial position of the solutes at \( z = 21 \) Å and 1 Å, respectively.

Table 5.1  The power \( n \), the slope, and the time interval for calculation of the power and the slope, corresponding to Figure 5.1.

<table>
<thead>
<tr>
<th>Solutes</th>
<th>( n )</th>
<th>Slope</th>
<th>Time (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (interface)</td>
<td>1.15</td>
<td>0.93</td>
<td>From 200 to 410</td>
</tr>
<tr>
<td>Ammonia (interior)</td>
<td>0.79</td>
<td>1.20</td>
<td>From 400 to 520</td>
</tr>
<tr>
<td>Carbon dioxide (interface)</td>
<td>0.81</td>
<td>0.57</td>
<td>From 170 to 460</td>
</tr>
<tr>
<td>Carbon dioxide (interior)</td>
<td>0.77</td>
<td>0.13</td>
<td>From 310 to 526</td>
</tr>
</tbody>
</table>

\( ^a \): The interface refers to the initial position of the solute at \( z = 21 \) Å.

\( ^b \): The interior refers to the initial position of the solute at \( z = 1 \) Å.
Table 5.2  Diffusion coefficients and displacement of ammonia and carbon dioxide in the POPC bilayer.

<table>
<thead>
<tr>
<th>Solutes</th>
<th>$D$ (cm$^2$/s)</th>
<th>Overall displacement (Å)$^a$</th>
<th>Displacement (Å) during the time interval $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (interface)</td>
<td>$1.54 \times 10^{-5}$</td>
<td>31.2</td>
<td>35.2</td>
</tr>
<tr>
<td>Ammonia (interior)</td>
<td>$2.00 \times 10^{-5}$</td>
<td>25.4</td>
<td>23.1</td>
</tr>
<tr>
<td>Carbon dioxide (interface)</td>
<td>$9.50 \times 10^{-6}$</td>
<td>21.4</td>
<td>22.8</td>
</tr>
<tr>
<td>Carbon dioxide (interior)</td>
<td>$2.20 \times 10^{-6}$</td>
<td>13.7</td>
<td>11.2</td>
</tr>
</tbody>
</table>

$^a$: overall displacement for simulation time 1 ns.

$^b$: average displacement for the time interval specified in Table 5.1.
Table 5.3  The Lennard-Jones collision diameters ($\sigma_{d\theta}$) of ammonia and carbon dioxide.

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Collision diameter (Å)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>2.6</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>3.3</td>
</tr>
</tbody>
</table>

$^a$: Reference [114].
Figure 5.2  Log(MSD) vs. log(time) curves of ammonia and carbon dioxide in the AmtB protein embedded in the POPC bilayer during 2.5 ns NVT dynamics.
Table 5.4  The power $n$, the slope, and the time interval for calculation of the power and the slope, corresponding to Figure 5.2.

<table>
<thead>
<tr>
<th>Solutes</th>
<th>$n$</th>
<th>Slope</th>
<th>Time (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>0.9</td>
<td>0.0011</td>
<td>From 44 to 460</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>1.1</td>
<td>0.0049</td>
<td>From 680 to 1740</td>
</tr>
</tbody>
</table>
Table 5.5 Diffusion coefficients and displacement of ammonia and carbon dioxide.

<table>
<thead>
<tr>
<th>Solutes</th>
<th>$D$ (cm$^2$/s)</th>
<th>Overall displacement (Å)$^a$</th>
<th>Displacement (Å) during the time interval $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia$^a$</td>
<td>$1.83 \times 10^{-8}$</td>
<td>2.44</td>
<td>2.35</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>$8.17 \times 10^{-8}$</td>
<td>7.20</td>
<td>4.90</td>
</tr>
</tbody>
</table>

$^a$: overall displacement for simulation time 2.5 ns.

$^b$: average displacement for the time interval specified in Table 5.4.
Ammonia and all ND1
Ammonia and all NE2
Figure 5.3  Radial distribution functions (RDFs) between ammonia and the \( \delta \) and \( \varepsilon \) nitrogen atoms in different histidine residues.

(a) RDF between ammonia and all \( \delta \) and \( \varepsilon \) nitrogen atoms in all histidine residue of the AmtB protein; (b) RDF between ammonia and the \( \delta \) and \( \varepsilon \) nitrogen atoms in His\(^{168}\); (c) RDF between ammonia and the \( \delta \) and \( \varepsilon \) nitrogen atoms in His\(^{318}\).
6. CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

The diffusion coefficients of ammonia through the DPPC and the POPC bilayers are in good agreement as being on the order of $10^{-5}$ cm$^2$/s. However, the calculated diffusion coefficient of ammonia through the POPC bilayer is comparable to the local diffusion coefficient reported in the headgroup region of the DPPC bilayer [22]. The calculated diffusion coefficient does not have any restraints on the displacement of ammonia. The diffusion coefficient of carbon dioxide is on the order of $10^{-6}$ cm$^2$/s. This is attributed to the size of the molecule and explained by the theoretical relationship between the diffusion coefficients of nonpolar and nonreacting gas molecules and the collision diameter of a LJ potential.

The diffusion coefficients of ammonia and carbon dioxide through the AmtB channel
protein are on the order of $10^{-8}$ cm$^2$/s. The lowered rate of the diffusion through the AmtB channel protein is attributed to lack of driving force for the solute molecules to overcome the energy barrier and confinement of the solutes within the free volume in the transport protein. However, the diffusion coefficient of carbon dioxide is higher than that of ammonia since one of the three carbon dioxide molecules is diffused out to the interface between the protein and the lipid bilayer. The reason for less strong association between carbon dioxide and the two conserved histidine residues is explained by the RDFs.

The RDFs between the solutes and the $\delta$ and $\epsilon$ nitrogen atoms of the histidine residues are calculated. The preferential association between ammonia and the $\delta$ and $\epsilon$ nitrogen atoms in the histidine residues is generally 10 to 30 times stronger than that between carbon dioxide and the $\delta$ and $\epsilon$ nitrogen atoms. This provides evidence for a free diffusion of the one carbon dioxide molecule allowed to diffuse within the protein. Furthermore, the degree of association tells us that carbon dioxide may not play a role of a substrate for the AmtB channel protein. That conclusion corresponds to which Kim et al. found that the substrate for the AmtB channel protein is ammonia and the substrate for the Rh-protein is carbon dioxide [35].

For future research direction, the transport mechanism of the solutes through the protein needs to resolve difficulties of the retardation of diffusion within the channel due to bulky side chains residing near the channel vestibule. In the MD simulations, neither the solutes nor water molecules had a chance to pass through the channel. A force field that can handle protonation and deprotonation between ammonia and ammonium cation is to be used because of the nature of the presence of ammonia and ammonium ion in biological systems. The protonation and deprotonation of the ammonia and ammonium cation is the key to transport of ammonia through biological membranes. The membrane-protein complex only
contained neutral ammonia molecules in the protein channel due to the limit of the CHARMm force field that cannot handle protonation and deprotonation of molecules. These phenomena can be effectively simulated by using a force field that can handle quantum mechanical calculations for protonation with the rest of the system being described through molecular mechanical calculations [38]. To effectively show diffusion of the solute molecules requires the introduction of a driving force that can overcome the hindrance of movement of the solutes.

REFERENCE


[26] D. Bemporad, *Computer simulation of biological membranes and small molecule*


[65] Supporting information of [31], available at

http://www.pnas.org/cgi/content/full/0406475101/DC1, accessed 1/27/05.


APPENDIX

CHARMm residue topologies of ammonia and carbon dioxide

MASS  13 HN0  1.00800 H ! ammonia hydrogen from toppar_amines.str
MASS  61 N0  14.00700 N ! ammonia nitrogen from toppar_amines.str

RESI AMM1  0.000  ! Ammonia
GROUP
ATOM N1  N0  -1.125
ATOM H11  HN0  0.375
ATOM H12  HN0  0.375
ATOM H13  HN0  0.375
BOND N1  H11  N1  H12  N1  H13
IMPR N1  H11  H12  H13
IC H13  H11  N1  H12  0.0  0.0  180.  0.0  0.0
CHARMm parameters for ammonia and carbon dioxide

BONDS
!V(bond) = Kb(b - b0)**2
!Kb: kcal/mole/A**2
!b0: A
!atom type Kb b0
N0  HN0  455.500  1.0140
    ! All-Atom amine parameters, toppar_amines.str
CST OST  937.960  1.1600
    ! toppar_co2.prm from c31b1

ANGLES
!V(angle) = Ktheta(Theta - Theta0)**2
!V(Urey-Bradley) = Kub(S - S0)**2
!Ktheta: kcal/mole/rad**2
!Theta0: degrees
!Kub: kcal/mole/A**2 (Urey-Bradley)
!S0: A
!atom types Ktheta Theta0 Kub S0
HN0 N0 HN0 29.000 107.1

! All-Atom amine parameters, toppar_amines.str
OST CST OST 3000.00 180.0000

! toppar_co2.prm from c31b1

IMPROPER
!V(improper) = Kpsi(psi - psi0)**2
!Kpsi: kcal/mole/rad**2
!psi0: degrees
!note that the second column of numbers (0) is ignored
!atom types Kpsi psi0
N0 HN0 HN0 HN0 15.0000 0 120.0000

! All-Atom amine parameters, toppar_amines.str
NONBONDED nbxmod 5 atom cdiel shift vatom vdistance vswitch -
cutnb 14.0 ctofnb 12.0 ctonnb 10.0 eps 1.0 e14fac 1.0 wmin 1.5
!adm jr., 5/08/91, suggested cutoff scheme
!V(Lennard-Jones) = Eps,i,j[(Rmin,i,j/ri,j)**12 - 2(Rmin,i,j/ri,j)**6]
!epsilon: kcal/mole, Eps,i,j = sqrt(eps,i * eps,j)
!Rmin/2: A, Rmin,i,j = Rmin/2,i + Rmin/2,j
!atom ignored epsilon Rmin/2 ignored eps,1-4 Rmin/2,1-4
N0 0.000000 -0.070000 1.980000

! All-Atom amine parameters, toppar_amines.str
HN0 0.000000 -0.012000 0.870000

! All-Atom amine parameters, toppar_amines.str
CST 0.000000 -0.058000 1.563000

! toppar_co2.prm from c31b1
OST 0.000000 -0.165000 1.692000

! toppar_co2.prm from c31b1