CHAPTER 1

INTRODUCTION

1.1 Cell membrane

Cell membrane or plasma membrane is close to the cell wall. It is a smooth or folded back and forth for size expansion into the cell membrane called mesosomes which the function in communication between cell to cell by controlling the water, nutrients various metal ions and specific molecules what goes in and out of across the cells between inner cell and outer cell.[1] Therefore cell membranes are associated with a variety of mechanical supports for cellular processes such as cell adhesion, ion conductivity and cell signaling.[1-2] Cell membrane is a selectively permeable membrane that encircles the cytoplasm of living organisms to protect the intracellular organelle from its extracellular surroundings. The cell membrane constitutes of two major compounds that are lipids and proteins. Especially the common lipids in most membranes are phospholipid. According to the "fluid mosaic model", they are arranged as a bilayer (Figure 1.1). They are amphipathic molecules arranged in a way that the hydrophilic heads are exposed to water region while the lipophilic tails face each other. Membrane proteins, on the other hand, are embedded in double layer of phospholipid. One of the most widely studied proteins that are embedded in the lipid bilayer is called membrane protein channels.[3]

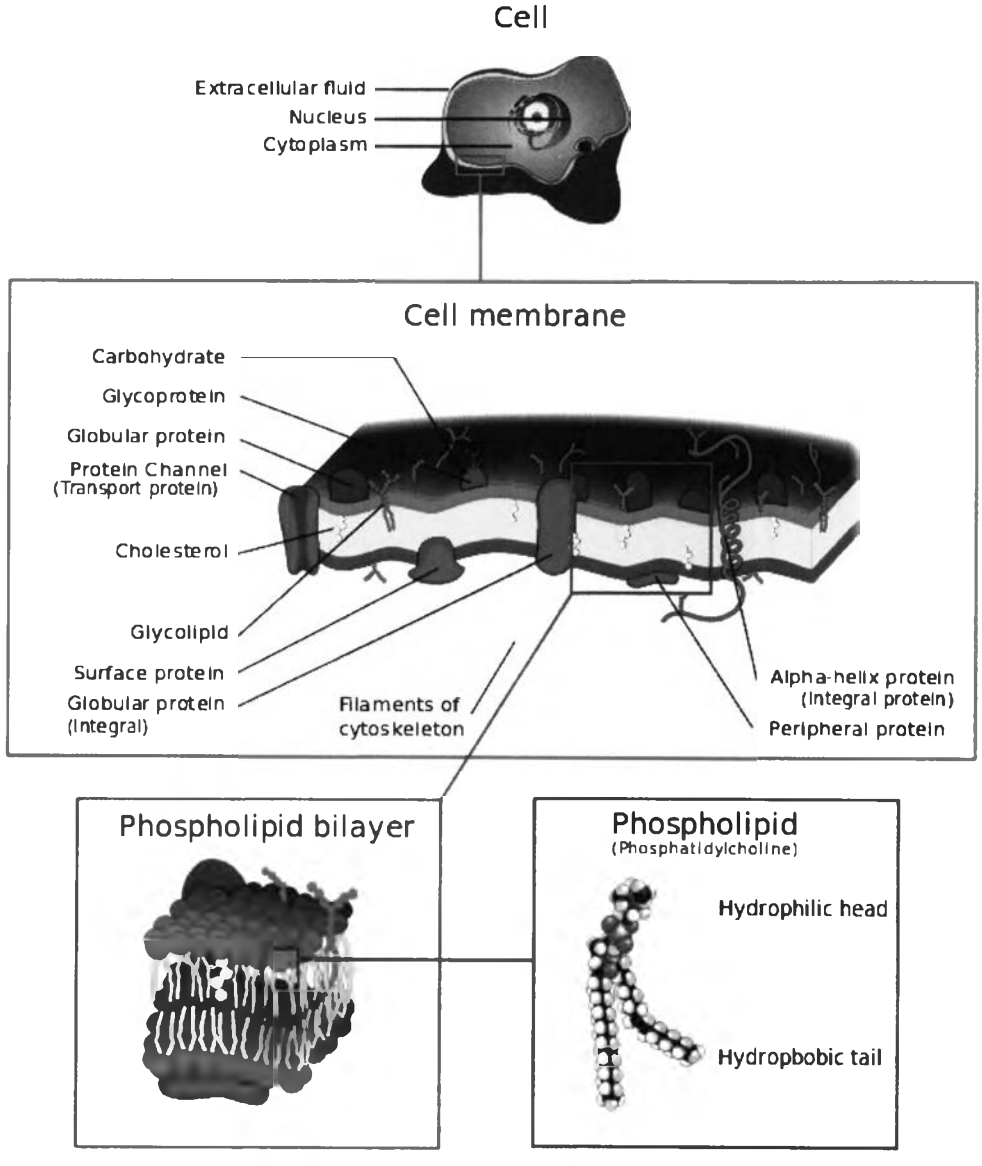

FIGURE 1.1 The fluid mosaic model representation of cell membrane or plasma membrane structure.[3]

1.2 Ion channel

Ion channels are a large integral membrane protein complex found in all living cells and located within the plasma membrane of nearly all cells and many intracellular organelles.[4] They present one of a major classes of membrane proteins in membrane biology. Ion channels are selectively permeable to specific types of ion species. Generally, they are described as narrow, water-filled tunnels that allow only ions of a certain size and/or charge to pass through. Their functions of ion channel include signal transduction and regulation of many physiological processes, maintaining the resting membrane potential and modulating electrical excitability in neuron and muscle cells.[5] They may be opened or closed in response to different types of stimuli such as chemical or electrical signals, temperature, or mechanical stimuli.[6]

There are many different types of ion channels which may be classified by different properties (Figure 1.2). The important features used for ion channel classification are the gating mechanism and the ion selectivity. For voltage-gated ion channels, they open and close the pore of channel in respond to changes in membrane potential between the inside and outside of cells.[7] The ligand-gated ion channels are the class that has ligand-binding domain. The binding of small ligand molecules such as acetylcholine, dopamine or serotonin etc. can trigger pore of the channel to change the conformation from close to open states for instance ATP-gated channels.[8] Mechanosensitive (Ms) ion channel is a class of ion channels that can be activated by mechanical force such as pressure, sound, touch and osmotic pressure.[9] For example, a mechanosensitive ion channel of large conductance (MscL) is activated by changes in osmotic stretch.[10]

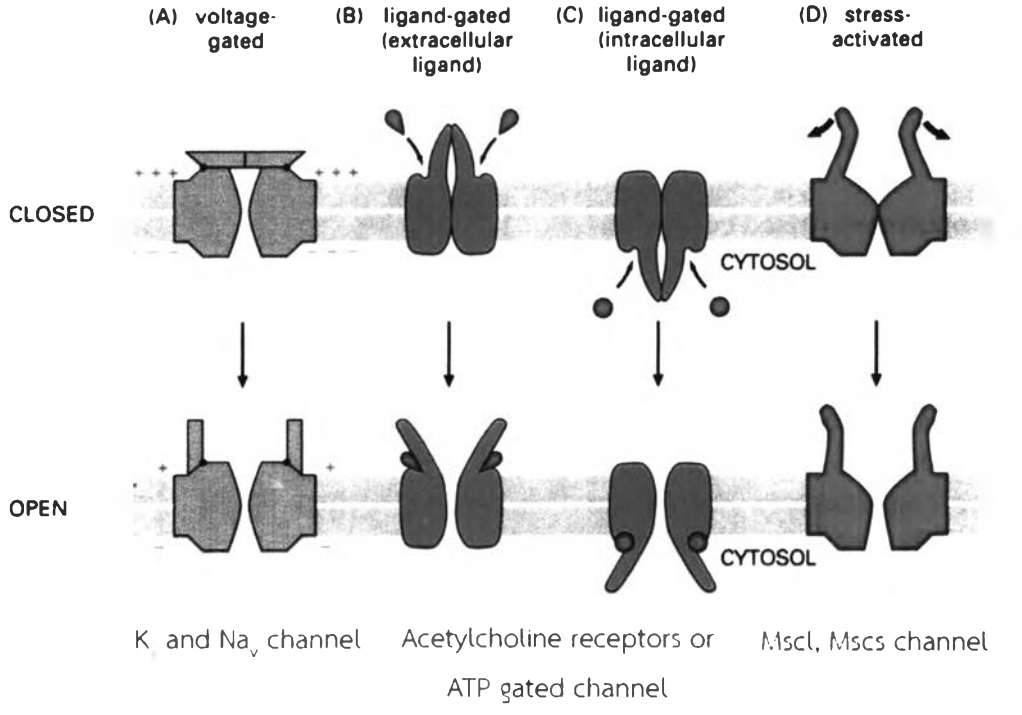

FIGURE 1.2 Classification of ion channels based on external stimuli. From left to right: voltage-gated ion channels, ligand-gated (extracellular ligand) ion channels, ligand-gated (intracellular ligand) ion channels and mechanically gated ion channels.[11]

1.3 Mechanosensitive channel

Mechanosensitive ion channels (Ms) are pore-forming membrane proteins that are activated through physical forces such as pressure, sound, touch and osmotic pressure, by transducing such mechanical stimuli into electrochemical signals. It has been found that high blood pressure, heart rate abnormalities and some neurological diseases are associated with the deformed of this protein.[12] The Ms protein acts as a signal transducer for mechanical, electrical or chemical. They are ubiquitous membrane proteins that are found both in eukaryotes and prokaryotes.[2]

Single cell organisms that often live independently control the balance of osmotic pressure between the inner and outer membrane system called homeostatic. Environment around cells can be changes at any time. So turgor pressure caused by osmotic pressure changes could harm to cells if the system doesn't have control such as the case of hypo-osmotic system. Water enters the cells and increases the size of turgor pressure. If the control cells system imbalances, cells swell and eventually rupture. In contrast. the hyper-osmotic system, cells control system the amount of water expelled from the cell to prevent the loss of too much water. Ms channels play an important role in controlled the balance of osmotic pressure in cells. According to their physiological function, Ms channels are now recognized as a "biological emergency release valve".[13]

The activities of mechanosensitive channel have been reported under a various conditions. These activities observed in bacterial native membranes were athletic and of relatively large conductance. All of these can be classified according to their single-channel properties on conductivity[2] as MscL, MscS or MscM. MscL, which the normally has a conductance on the order of 3.0 nanosiemens (nS). MscS (mechanosensitive channel of small conductance), a smaller conducting channel activity (about 1nS) opens at stimuli less than that required for MscL and has been referred to as MscS. MscM or mechanosensitive channel of minimum conductance has been yet smaller conductance less than equal to 0.3 nS with the gene has yet to be identified. It also has MscK (the potassium-dependent mechanosensitive channel) which are similar MscS channel but in opening of channel must be stimuli by pressure and also the higher concentrations of ions outer cell such as K^+, $NH4^+$, Rb^+ or Cs^+.[14]

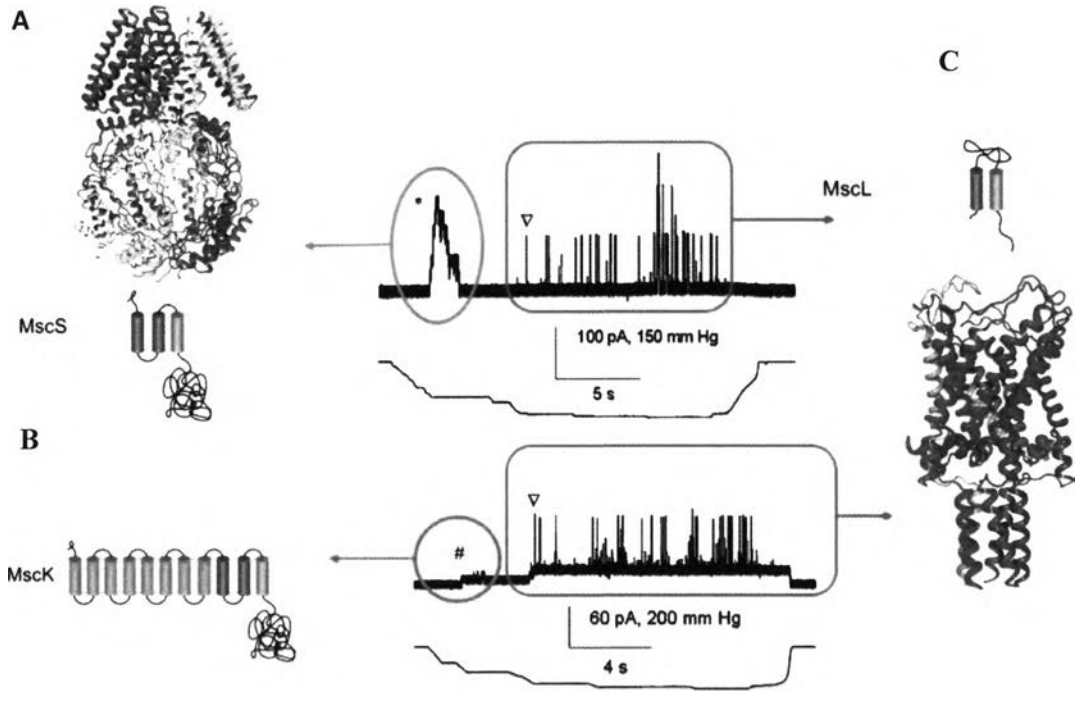

FIGURE 1.3 Typical current traces of MscS (A) and MscK (B) in the presence of MscL(C). Recordings were generated from patches derived from E. coli giant sphero plast at−20 mV. MscL is a homopentamer in which each monomer has two TM segments. The open state is characterized by a very large single channel conductance (~3.5 nS) and activation tensions close to the lytic limit of biological membranes. MscS, as originally described, is actually the result of two distinct gene products, YggB, which underlies MscS proper, and KefA, which is now known as MscK because of the role of potassium ions in modulating activity. Both channels have similar single channel conductances (~1 nS) and are activated at intermediate tensions. MscS activity also shows a distinctive inactivation/adaptation phenomenon.[14]

1.4 Mechanosensitive channel of large conductance

Microbial cells constitutively express the large conductance of mechanosensitive channel (MscL) appears to play as a "safety valve" by opening a large pore to release a high pressure of cells due to osmotic stress. The primary role of MscL is thought to protect bacteria from lysis upon sudden osmotic shock. MscL has a relatively large conductance and therefore allows the passage of ions, water, peptides or small proteins during channel gating.

MscL is a widely-studied model system for understanding the mechanosensation and mechanotransduction, a process of which protein senses and transduces mechanical stimuli into electrophysiological activity. Generally, the study involves how lipid regulates protein conformational changes. Crystallographic structure of MscL from *Mycobacterium tuberculosis* (mtbMscL) by X-ray crystallography has provided insight into the structure architecture of mechanosensitive channels.[15] In mtbMscL each of these chains is known to be made of 136 residues. MscL is a homopentameric ion channel of which each subunit is composed of two transmembrane helical segments (TM1 and TM2), N- and C-terminal helices and a periplasmic loop. TM1 (residue 14 to 43) and TM2 (residue 72 to 107) are connected by a periplasmic loop and the N-terminal helix (residue 1 to 13) lies almost parallel to membrane leaflet of the intracellular side. The C-terminal domain (rersidue 44 to 71) also forms pentameric helical bundle in cytoplasm. The pathway of ion conduction of the MscL channel is formed by the inner TM1 transmembrane helices with their hydrophilic residues. In the crystallized closed state, the constriction forms a hydrophobic gate with radius about 2 Å.[12]

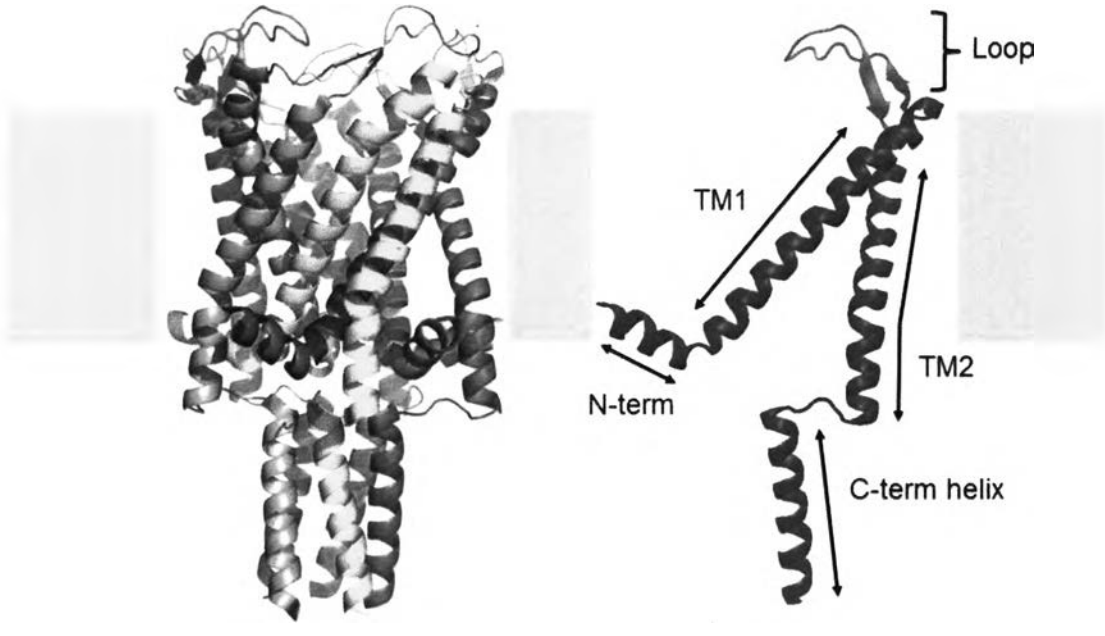

FIGURE 1.4 Schematic representation of (A) the pentamer structure and (B) the monomer structure of mtbMscL. Three structure domains including transmembrane helices (TM1 and TM2), N-terminal and C-terminal in cytoplasmic domains are illustrated.[12]

1.5 Osmotic regulation of cell bacteria

Under physiological conditions in cells, almost all bacterial cells have evolved a protective mechanism to changes surrounding cells and variations in external osmolarity, particularly, the response to high osmolarity in nature of solute accumulated. The increase of membrane tension arises from the rapid flow of water in cells system, which is associated with changes in the bacterial cells from the surrounding environment. The cell membrane has a osmotic pressure that can cause serious problems for a cell. Because the cell is filled with salts, sugars, proteins, and other molecules, it will almost always be hypertonic to water. This means that osmotic pressure should produce a net movement of water into a typical cell that is surrounded by water. If that happens, the volume of a cell will increase until the cell becomes swollen. Eventually, the cell may burst like an overinflated balloon.[13] Thus cellular responses to changes in the osmotic environment to maintain a balance in the cells.[16] Figure 1.5[13], shows the effect from hypo-osmotic stress, when bacterial cells are exposed to a growing in low osmolarity medium accumulating 300- to 400-mM ions such as potassium (red dots) and glutamate (blue dots) through transporters from low osmolarity (hyperosmotic shock) to one of high osmolarity initially causes efflux of water and cell shrinkage and to prevent loss of the turgor pressure necessary for growth ,maintaining level of turgor pressure and maintaining cell shape, cells accumulate more potassium and glutamate as well as compatible solutes (white dots). After transfer of cells from high to low osmolarity (hypo-osmotic shock), MS channels in the cytoplasmic membrane are activated by the rapid increase in membrane tension caused by the entry of water by the activation of Ms channel gradually opening up to the membrane tension at the save time from close state to open state through intermediate state. These large-diameter nonspecific channels mediate the immediate release of ions and compatible solutes, saving cells from lysis. If the channels are absent or fail to open, the influx of water generates high turgor pressure, which leads to the lysis of the cell when the pressure exceeds the mechanical strength of the wall.[17]

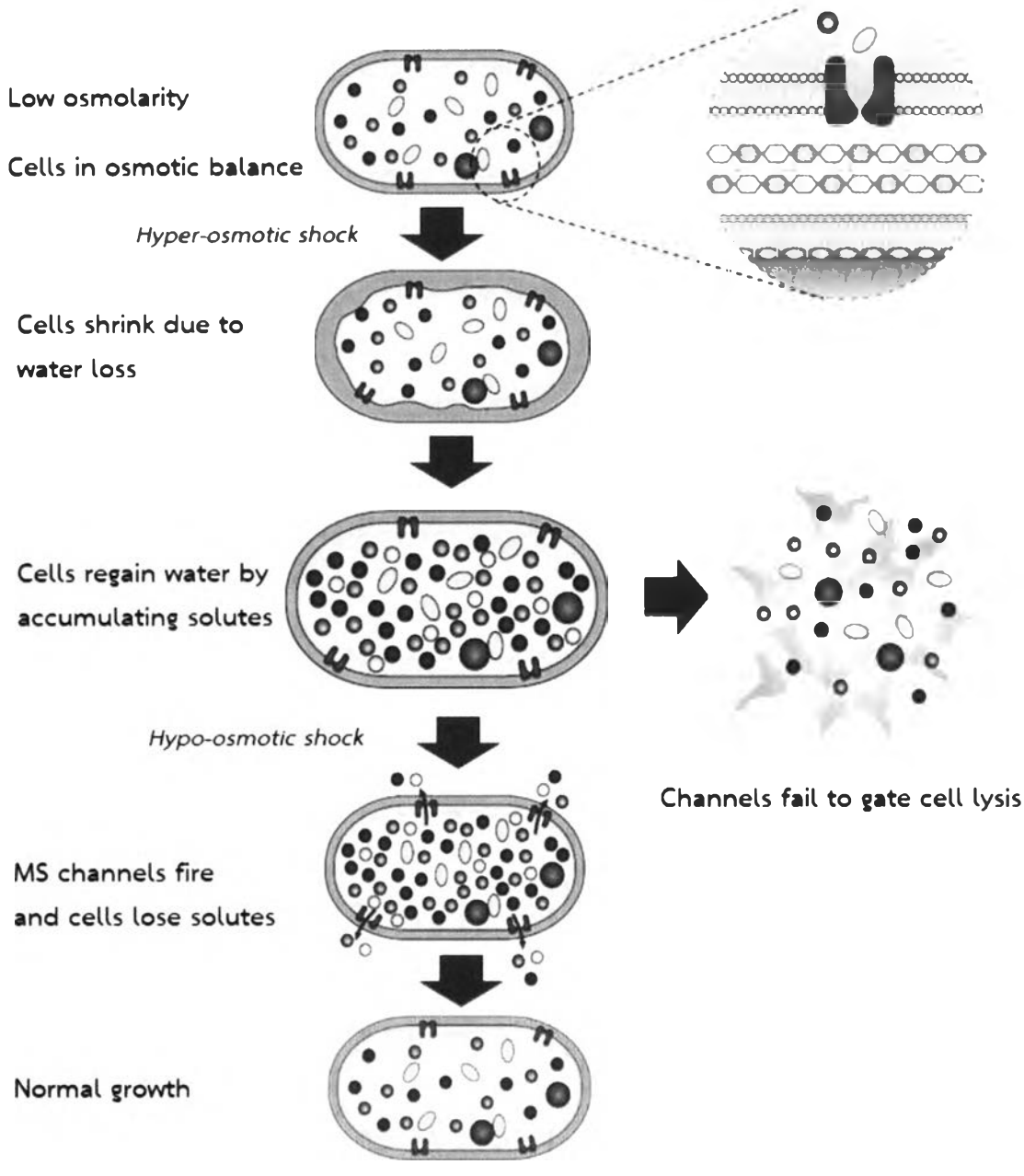

FIGURE 1.5 Cellular responses to changes in the osmotic environment.

1.6 Electron Paramagnetic Resonance and Spin Labeling

The site-directed spin labeling (SDSL) in combination with electron paramagnetic resonance (EPR) spectroscopy have been shown as an efficient tool to the elucidation of structural and conformational dynamics of biomolecules under physiological state of the system.[18] The technique is applicable to soluble molecules, e.g., proteins and nucleic acids, as well as to membrane proteins either solubilized in detergent or embedded in the lipid bilayer. The size and complexity of the system under investigation is almost arbitrary. SDSL involves attachment of a spin label side chain by covalent, which contains a stable unpaired electron, to a specific site on a protein The most commonly used spin label is the sulfhydryl-specific nitroxide,2,2,5,5-tetramethyl-l-oxyl-3-methyl methanethiosulfonate (MTSSL).[19]

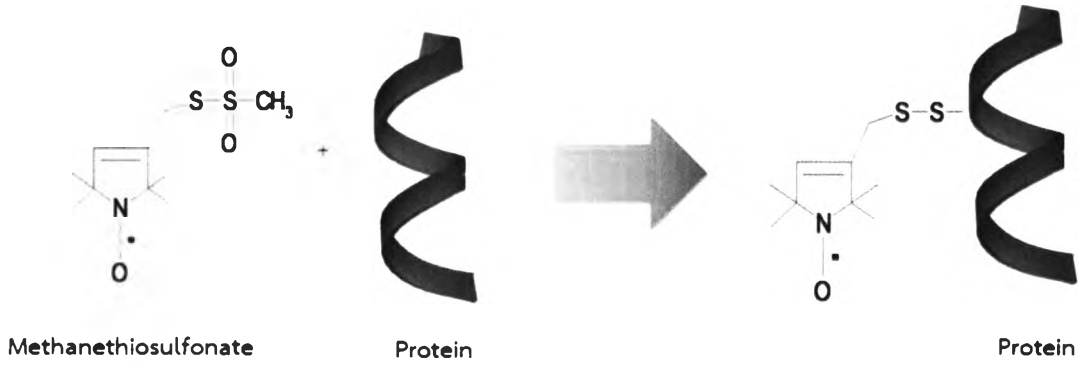

FIGURE 1.6 Reaction of the methanethiosulfonate spin label (MTSSL) with the sulfhydryl group of a cysteine side chain, generating the spin label side chain.

Spin labeling is relied on the reaction of sulfhydryl groups of cysteine residues which are engineered into the protein under investigation through site-directed mutagenesis. This approach usually requires that the target proteins possess only cysteine residues. This spin label is bound to the protein by formation of a disulfide bond to the sulfhydryl group of the cysteine.[20]

1.7 Literature reviews

1.7.1 Lipid-driven conformational changes of MscL: Hydrophobic mismatch and geometric consequences of bilayer

Biological membrane is kind of amphipathic layer that acts as a barrier within or around a cell.[21] In most case, it is a lipid bilayer, composed of a double layer of lipid molecules and proteins that may constitute close to 50% of membrane content. The proteins embedded inside lipid bilayer could have different hydrophobic length that the interaction of integral proteins with the lipids inside membrane bilayer is of great importance for membrane function.[22]

Hydrophobic mismatch arises from a difference in the hydrophobic thickness of lipid bilayer and a transmembrane protein segment embedded in bilayer, and is thought to play an important role in the folding, stability and function of membrane proteins, Caused to the aggregation of transmembrane proteins embedded in bilayer which hydrophobic mismatch is important for many biological processes.[23]

In 2002. Perozo,[21] and et al, found that changes in the physical properties of lipid bilayer such as the thickness of the membrane and asymmetry of lipids affect to ecoMscL and conformation changes from close to open state via intermediate state. ecoMscL channel reconstituted with phosphatidylcholine (PC18) stabilized the closed conformation. Phospholipid with the shorter of hydrophobic carbon tale such as PC12, PC14 and PC16 has shifted the population of the closed state to an intermediate conformation. This is due to an influence of hydrophobic mismatch and the geometric consequences of bilayer intrinsic curvature. Finally, Perozo et al was able to trap the stable open-state conformation of ecoMscL channel by reconstitution the protein in asymmetric phospholipid "lysophosphatidylcholine (LPC)".

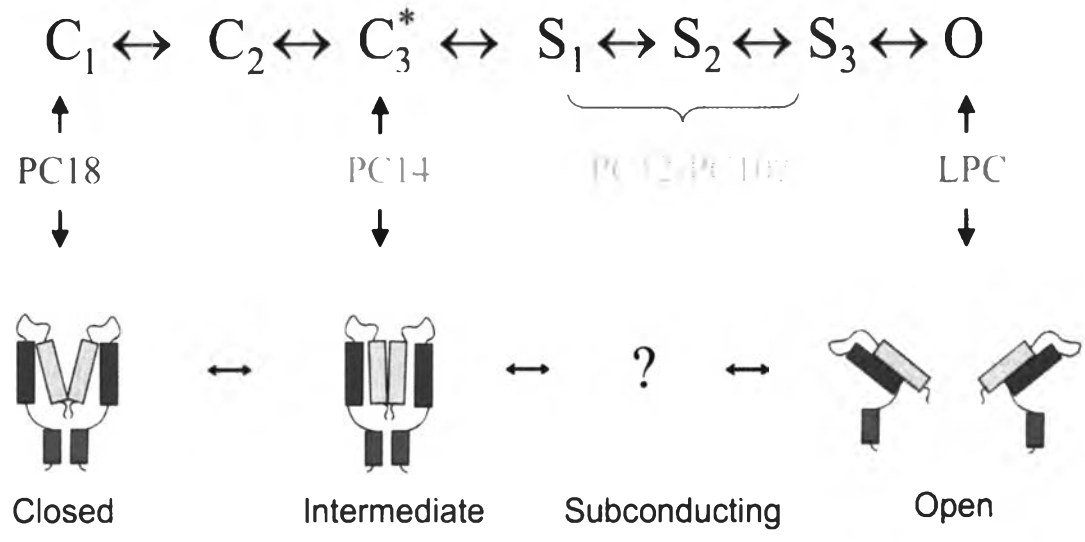

FIGURE 1.7 A model depicting the evolution of structurally distinct conformations during MscL gating. The top row represents a hypothetical sequence of kinetic events MscL undergoes on its way to the fully open state. Manipulating the lipid environment surrounding the channel can trap at least three of these distinct conformations: (i) the closed state is stable in PC18, (ii) PC14 stabilizes a closed conformation further along the kinetic path and (iii) the fully open state can be locked by addition of conical-shaped lipids (LPC) on one leaflet of the bilayer. The possibility remains of stabilizing additional intermediates (such as subconducting states) using even shorter (but more unstable) bilayers.[21]

Hydrophobic matching and bilayer curvature stress are not independent properties. Figure 1.8 describes a gating mechanism of MscL which is associated with a conformational transition from closed to fully open state.[24] An intermediate state is described by a state during transition between these two states. A previous structural study showed the influence of lipid acyl chain length and geometry on structure stability of MscL, When used 1, 2-dioleoyl-sn-glycero-3-phosphocholine (PC18 or 18 carbon) that ecoMscL stabilizes the closed state conformation and when reduced of acyl chain length in phospholipid tale such PC16, PC14 or PC12, the ecoMscL stabilized in intermediate state due to the effect of hydrophobic mismatch. When modified by the incorporation of lysophosphatidylcholine (LPC) found that ecoMscL stabilized in the fully open state.

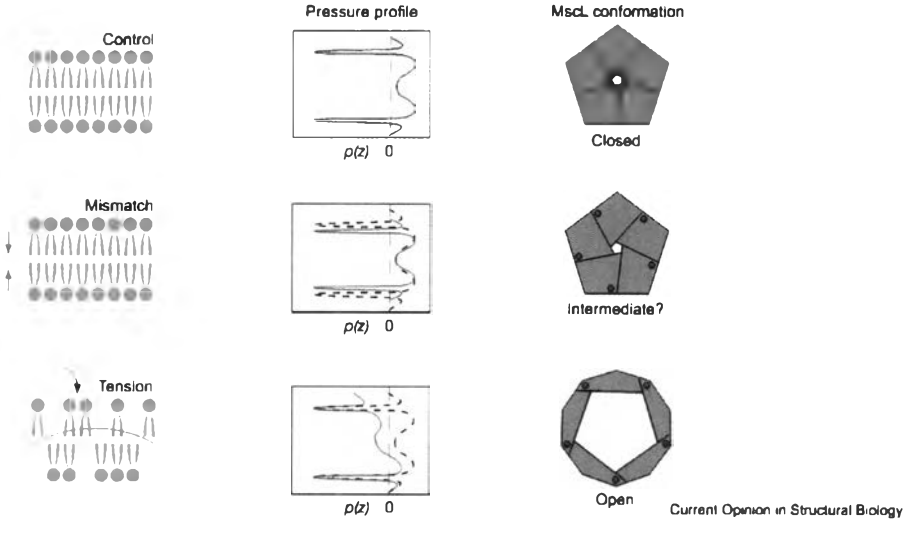

FIGURE 1.8 Physical changes in the lipid bilayer and the structural state of MscL. Shown in each case are diagrams of the type of bilayer perturbation (left), the estimated TM pressure profile (middle) and the corresponding functional state of MscL (right). (a) An unperturbed (control) bilayer stabilizes the channel in its closed conformation.(b) Reconstitution of MscL into bilayers of different thickness compresses/expands the pressure profile and biases the threshold of activation through hydrophobic mismatch, possibly stabilizing an intermediate conformation of the channel.(c) Asymmetric incorporation of cone-shaped lipids (i.e. LPC) alters the pressure profile, favoring the fully open state.[2]

1.7.2 Structure refinement of membrane proteins based on SDSL-EPR

In 2008. Sompornpisut et al,[25] developed a method called PaDSAR (Pseudoatom Driven Solvent Accessibility Refinement) for membrane protein refinement based on SDSL-EPR technique. In this study, this method converts solvent accessibility (including oxygen accessibility and nickel ethylenediaminediacetate or NiEDDA accessibility) data into structural restraints used in molecular dynamics simulations. The restraints are enforced through interactions between a pseudo-atom representation of the covalently attached Nitroxide spin-label and virtual "solvent" particles corresponding to O_2 and NiEdda in the surrounding environment. Interactions were computed using an empirical potential function, where the parameters have been optimized to account for the different accessibilities of the spin-label pseudo-atoms to the surrounding environment.

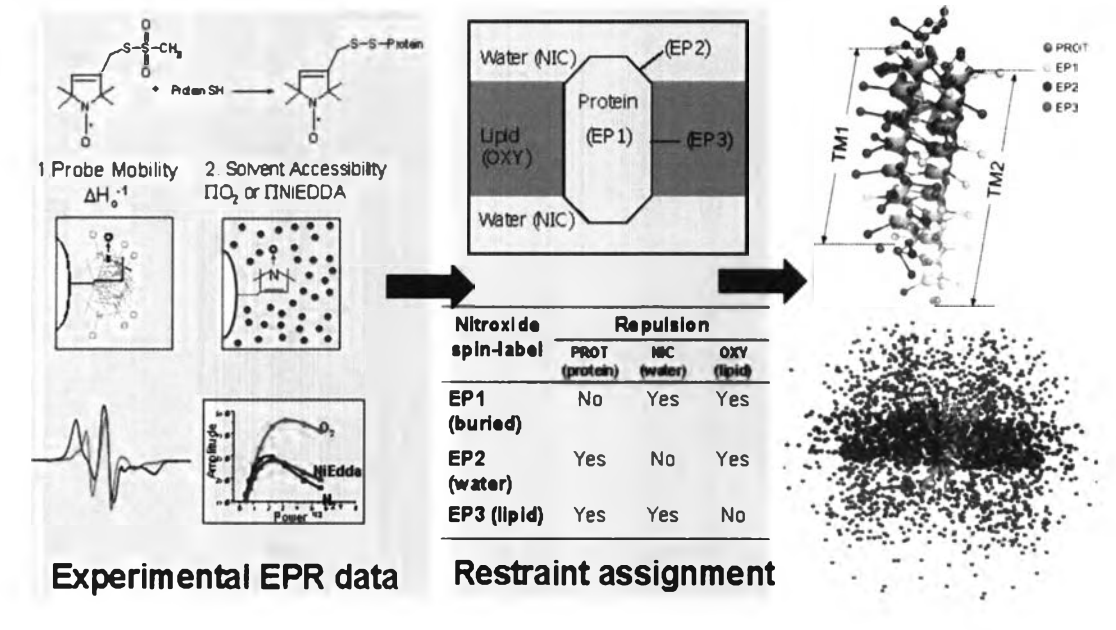

FIGURE 1.9 PaDSAR method based on data from SDSL-EPR technique to the structural refinement of membrane proteins through restrained molecular dynamics simulations.

1.8 Inspiration and objectives of this research

The study of ecoMscL in biological membrane could provide the fundamental basis for understanding how a protein senses the change in membrane tension and transduces to protein motion. The molecular mechanism underlying the mechanosensation requires detailed structure information of various conformational states associated with the gating. Unfortunately, the crystal structures of MscL from *Mycobacterium tuberculosis* (*mtbMscL*) is only available for the closed state. This study has been initiated with an inspiration of the extensive spectroscopic analysis by Perozo et al. He and his colleagues have rigorously assessed various conformational states of ecoMscL by means of SDSL-EPR technique. In their study, site-directed cysteine mutants were generated for residues 14–43 and 72–107 in ecoMscL, covering the TM1 and TM2 helices. Figure1.10, shows the SDSL-EPR data (mobility, O_2 and NiEDDA accessibility) of ecoMscL reconstituted in PC18 (closed state) and in PC12 (an intermediate state) . The mobility data provide dynamics information related to backbone motions which depend on the length and flexibility of the linker between the nitroxide and the protein backbone and steric restraints of neighboring residues. In addition, the accessibility parameters (Π). provide information on orientation and position of proteins within the membrane.

EPR data of TM residues in MscL channel in PC18 and PC12

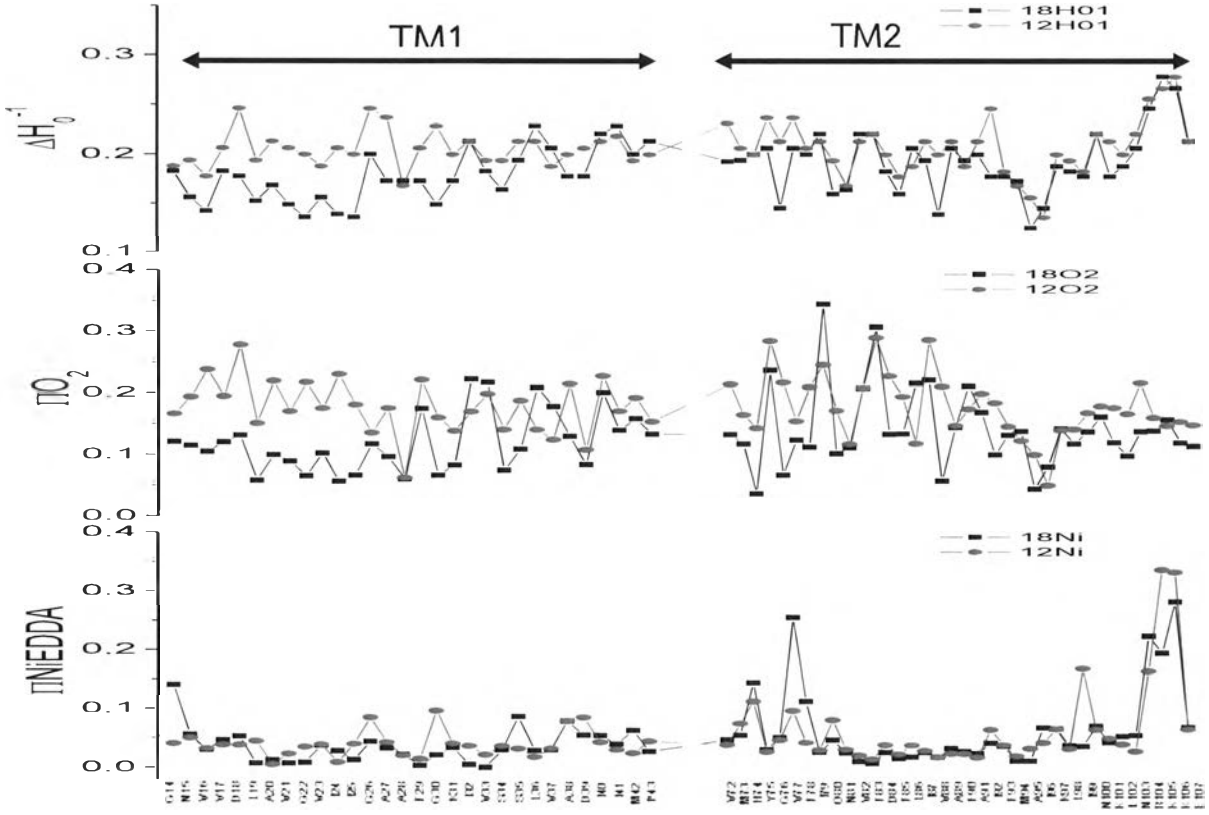

FIGURE 1.10 EPR data of TM residues in MscLchannel.

The available experimental data taken from the collaboration allows a possibility to investigate close conformation and intermediate conformation using PaDSAR, a specifically developed approach for modeling conformation changes of membrane protein using EPR restraints.

The main objectives of this study are:

1. To construct the close state model and intermediate state model of ecoMscL from PaDSAR.

2. To investigate structure and dynamic properties of closed and intermediate state models of ecoMscL by means of MD simulations.