MOLECULAR DYNAMICS STUDIES: THE INTERACTION OF ERYTHROMYCIN A WITH A SINGLE BASE SUBSTITUTION OF 50S RIBOSOMAL SUBUNIT

by

SITI ZURAIDAH BINTI MOHAMAD ZOBIR

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LIST OF ABBREVIATIONS

3D	three-dimensional
A	adenine
Å	angstrom (10^{-10}m)
AMBER	Assisted Model Building and Energy
C	cytosine
CG	conjugate gradient
CHARMM	Chemistry at Harvard Macromolecular Mechanics
DNA	deoxyribonucleic acid
D. radiodurans	*Deinococcus radiodurans*
EC	*Escherichia coli* numbering
ERYA	erythromycin A
fs	femtosecond
G	guanine
GAMESS	General Atomic and Molecular Electronic Structure System
GAFF	General Amber Force Field
GROMACS	Groningen Machine for Chemical Simulations
H-bond	hydrogen bond
H. marismortui	*Haloarcula marismortui*
K_d	dissociation constant
K	Kelvin
MD	molecular dynamics
MIC	minimum inhibitory concentration
MOPAC	Molecular Orbital PACkage
mRNA	messenger RNA
NPT	isothermal-isobaric ensemble
ns	nanosecond
NVT	canonical ensemble
O	oxygen
PDB	Protein Data Bank
ps	picosecond
PTC	peptidyl transferase center
PME	Particle Mesh Ewald
R.E.D.	RESP ESP charge Derive
RDF	radial distribution function
RESP	restrained electrostatic potential
RMS	root mean square
RMSD	root mean square deviation
RMSF	root mean square fluctuation
RNA	ribonucleic acid
rRNA	ribosomal RNA
S.erythreus	*Streptomyces erythreus*
SD	steepest descent
SPARTAN	computational approach software from Wavefuntion, Inc
TB	terabyte
U	uracil
VMD	Visual Molecular Dynamics

KAJIAN DINAMIK MOLEKUL: INTERAKSI ANTARA ERITROMISIN A DENGAN SUB -UNIT RIBOSOM 50S PENGGANTIAN TUNGGAL PADA SATU BES

ABSTRAK

Ribosom adalah tapak sintesis protein yang merupakan sasaran utama bagi antibiotik semulajadi dan sintetik dan salah satu daripadanya ialah makrolida. Makrolida tergolong dalam famili penting antibiotik klinikal yang selalu digunakan untuk merawat jangkitan oleh bakteria Gram-positif. Ribosom bakteria (70S) mengandungi dua sub-unit, sub-unit 30S dan 50S, dengan sub-unit yang lebih besar terdiri daripada 23S dan 5S rRNA yang juga membentuk pusat transferase polipeptidil (PTC). Secara umumnya, makrolida mengikat bersebelahan PTC berhampiran dengan bukaan terowong ribosom paling kecil, di mana polipeptida nasen keluar. Kegagalan tumbesaran sel akibat daripada pengikatan makrolida pada sub-unit besar ribosom telah banyak didokumentasikan kerana sintesis protein yang lengkap telah dihalang oleh penceraian pra-matang polipeptida nasen yang sedang bergerak keluar dari terowong ribosom. Akibat penggunaan makrolida secara meluas, ribosom bakteria juga telah menunjukkan rintangan terhadap pengikatan makrolida. Walaubagaimanapun, mekanisme sebenar makrolida terhadap rintangan ubat masih samar.

Objektif kajian ini adalah untuk mengetahui bagaimana interaksi yang berlaku antara eritromisin A (ERYA), ahli-14 cincin makrolida, dengan sub-unit besar ribosom yang mengandungi penggantian tunggal pada bes 2041 daripada adenina kepada guanins. Penggantian tunggal bes ini telah dilaporkan sebagai salah

satu cara merintang pengikatan makrolida. Simulasi dinamik molekul (MD) adalah kaedah terbaik yang digunakan dalam kajian ini untuk menjelaskan di peringkat molekular mengapa rintangan terjadi apabila berlakunya penggantian tunggal pada bes atau residu 2041 iaitu salah satu bes dalam PTC.

Dengan mengaplikasikan MD, keputusan menunjukkan terdapatnya interaksi yang persis dan kuat terbentuk melalui rangkaian ikatan hidrogen dan interaksi hidrofobik antara ERYA dan bes-bes dalam PTC dan bukan di bes 2041. Kekuatan interaksi-interaksi ini telah membentuk ruang kosong di dalam terowong ribosom berhampiran residu-residu PTC lalu membenarkan molekul air untuk melalui terowong tersebut. Penemuan molekul air melalui terowong ini mungkin juga membenarkan polipeptida nasen untuk keluar daripada terowong ribosom dan mengakibatkan rintangan. Selain itu, tenaga pengikatan bebas terhasil daripada ikatan ERYA di bahagian PTC dikira melalui simulasi pendokkan molekul menggunakan struktur purata yang diprolehi daripada simulasi MD. Keputusan nilai keafinan daripada simulasi pendokkan molekul menunjukkan pengikatan ERYA dalam poket pengikatan adalah kurang baik apabila dibandingkan dengan sub-unit ribosom 50S liar.

MOLECULAR DYNAMICS STUDIES: THE INTERACTION OF ERYTHROMYCIN A WITH A SINGLE BASE SUBSTITUTION OF 50S RIBOSOMAL SUBUNIT

ABSTRACT

Ribosome, the site of protein synthesis, is a major target for natural and synthetic antibiotics and one of them is the macrolides. The macrolides belong to one of the most commonly used families of clinically important antibiotics to treat infections caused by Gram-positive bacteria. Bacterial ribosome (70S) composed of two subunits, 30S and 50S subunits and the larger subunit is made up of 23S and 5S rRNA that contains the peptidyl transferase center (PTC). The macrolides, in general, bind adjacent to the PTC near to the narrowest portion of the ribosomal tunnel, where the nascent polypeptide exits. It has been well documented that macrolides bind to large ribosomal subunit to cause cell growth arrest due to inhibition of protein synthesis by premature dissociation of nascent polypeptide from the ribosomal tunnel. Due to their extensive usage, bacterial ribosome has showed resistance towards the binding of macrolides. However, the exact mode of actions of macrolides towards drug resistance remains obscure.

The objective of the study is to find how the interactions erythromycin A (ERYA), a 14-membered ring macrolide, with a single base substitution from adenine to guanine on the large ribosomal subunit at 2041. This single base substitution has been reported of to be one of the resistance modes in the binding of macrolides. Molecular dynamics (MD) simulation is a great tool that was used in this study to explain at the molecular level why resistance happens when a single base substitution takes place at 2041, one of the residues in PTC.

By applying MD, results showed that persistent and strong interactions through hydrogen bonding network and hydrophobic interaction formed between ERYA and residues at PTC site and not at 2041. These strong types of interactions have created empty space inside the ribosomal tunnel near to PTC site to allow water molecules to pass through the ribosomal tunnel. The finding of water molecules to pass through this tunnel might also explain how nascent poplypeptide is allowed to escape the ribosomal tunnel, thus causing the resistance. Besides that, the free energy binding of ERYA towards the PTC site was also calculated via molecular docking simulation using the average structure from the MD simulation. The value of the binding affinity from the docking result showed that ERYA was less favorable to bind in the binding pocket when compared to the wild type of 50S ribosomal subunit.

CHAPTER ONE

INTRODUCTION

1.1 Statement of the Problem

Ribosome is one of the cytoplasmic granules in bacteria that are made of complexes of ribonucleic acids (RNA)s and proteins. It plays a very important role in protein synthesis by translating genetic codes into protein or polypeptide. Due to its important role, many antibiotics have been designed or naturally found to cause bacteria's death by interfering the protein synthesis that result in no protein, which is required carrying out all biological processes in bacteria, is being synthesized.

One of the safest and most clinically used groups of antibiotics is macrolides. The clinically active macrolides have 12- to 16-membered lactone ring and erythromycin A (ERYA) is the first macrolide to be introduced to treat infections. ERYA binds to 50S ribosomal subunit at the peptidyl transferase center (PTC) that is part of the ribosomal tunnel. The binding causes dissociation of premature newly synthesized polypeptide. However, over time, bacteria develop mechanism to be resistant in many ways and the exact mechanism how resistance happens is still obscure. ERYA has developed resistance towards binding to the 50S ribosomal subunit due to mutations. The mutation involves a single base substitution at residue 2041 in *Deinococcus radiodurans (D.radiodurans)*. A2041 is not conserved among all phylogenetic domains and in mitochondrial and cytoplasmic rRNAs of higher eukaryotes have G at position A2058EC *(E.coli* numbering). Residue 2058 in *E.coli*

is equivalent to 2041 in *D.radiodurans*. A2058GEC is the first nucleotide mutation to confer ERYA resistance.

Magnesium ion is known to be essential for stability and folding the large bacterial ribosome. In this study, two magnesium ions are present that might affect the binding affinity towards ERYA resistance.

The objective of this study is to investigate at the molecular level on how the single substitution of nucleotides from adenine (A) to guanine (G) at position 2041 of *D.radiodurans* contributes to the resistance exists in the binding of ERYA in 50S ribosomal subunit using molecular dynamics simulations (MD). The second objective is to investigate the role of magnesium ions that might contribute to the resistance of binding ERYA in the single base substitution of 50S ribosomal subunit. Finally, the last objective of the study is to calculate the free energy of binding between ERYA and 50S ribosomal subunit using molecular docking simulation approach. The theoretical value indicates the binding affinity between the ligand and the receptor. The PDB ID is 1JZY contains crystal structure of ERYA binds to 50S ribosomal subunit with three proteins and two magnesium ions.

1.2 Ribosome

Ribosome is the ribonucleoprotein complex that is responsible for translation of the genetic code, which is the last step in gene expression pathway, to produce proteins in all living organisms (Korostelev et al., 2008, Ban et al., 2000). It is well known that the bacterial ribosome composed of two unequal subunits; 30S and 50S subunits (Figure 1.1), which is a combined sedimentation coefficient (S) of 70S and both subunits perform different roles during protein synthesis (Nelson and Cox, 2004). Similar to other organisms, the bacterial ribosome, with molecular weight (MW) about 2.6 x 10^6Dalton (D), is two-thirds RNAs and one-third proteins and the larger subunit is approximately twice the molecular weight of the smaller subunit (Ban et al., 2000).

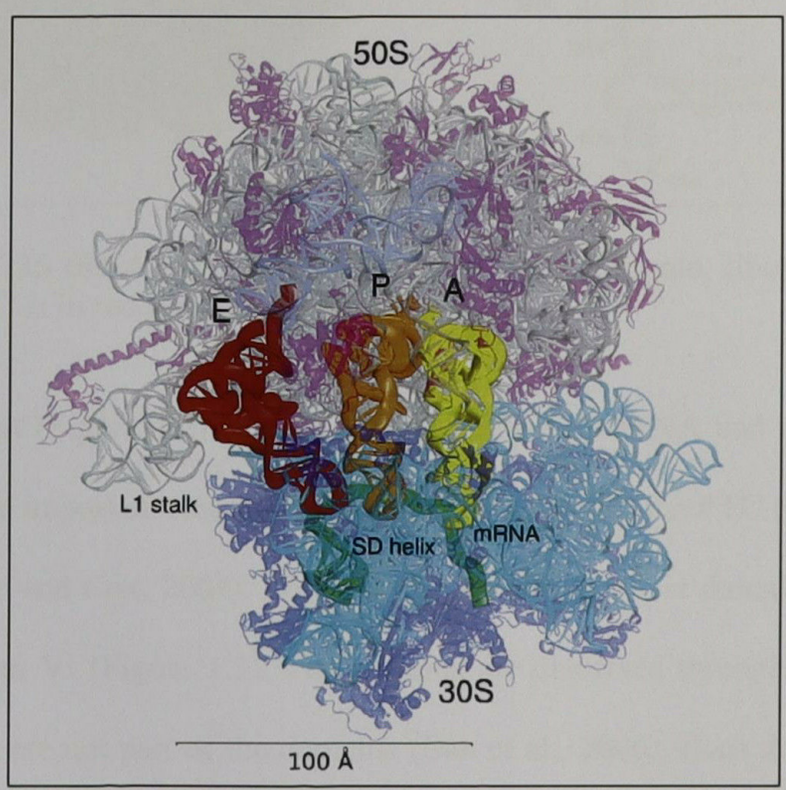


Figure 1.1: 70S ribosome with two subunits, 50S and 30S. E, P, and A sites are where protein synthesis takes place (Korostelev et al., 2008).

The smaller subunit, 30S, which contains 16S rRNA (about 1500 nucleotides) and more than 20 proteins, binds to the mRNA and is responsible for mediating the correct codon-anticodon interaction (Korostelev et al., 2008, Nelson and Cox, 2004). This interaction is also known as mRNA translation in order to determine the sequence of amino acids in the protein being synthesized (Steitz, 2008).

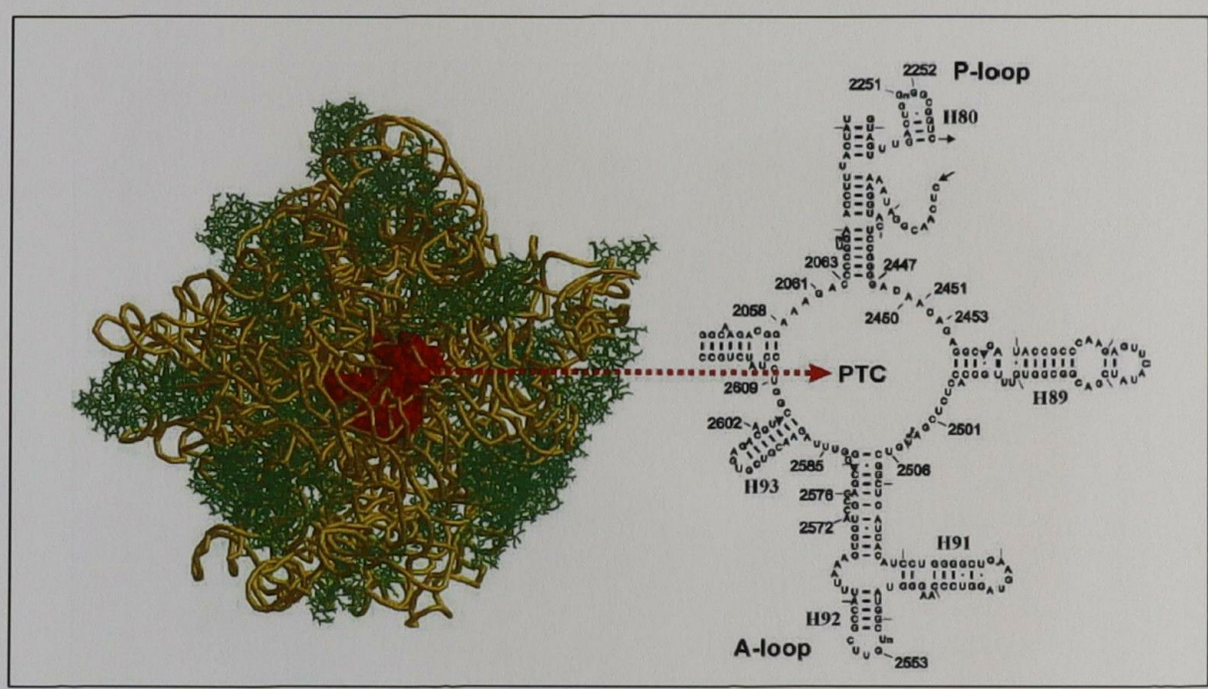


Figure 1.2: The 23S rRNA and 5S rRNA. They are shown in gold, ribosomal protein in green and PTC is in red (Polacek and Mankin, 2005).

The large subunit (Figure 1.2) is made up of 23S and 5S rRNA and more than 30 proteins with one important characteristic that it also contains the PTC (Korostelev et al., 2008, Nelson and Cox, 2004). The 23S rRNA consists of six domains, explicitly from Domain I to VI (Figure 1.3). The proteins are dispersed throughout the large subunit but they are not part of the domains (Ban et al., 2000). Each domain can be geographically explained starting from Domain I and II, which account for most of the back of the particle, Domain III is located at the bottom left region of the subunit in the crown view, most interface surface of Domain IV in 50S subunit contacts with

the 30S subunit, Domain V sits in between Domain IV and II in the middle of the subunit, and Domain VI is the smallest Domain in 23S rRNA (Ban et al., 2000).

Domain V is known to be thoroughly involved in peptidyl transferase activity of the ribosome in the PTC and PTC probably plays a crucial functional site in the ribosome (Ban et al., 2000, Vester and Garrett, 1988).

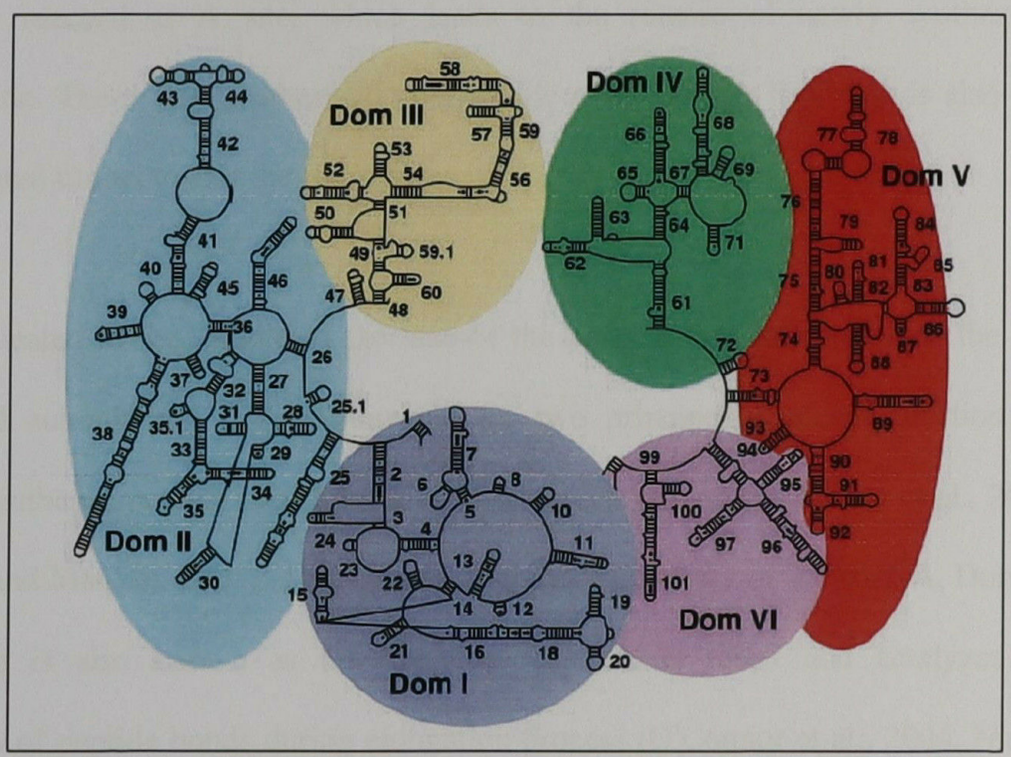


Figure 1.3: Domains I through VI of secondary structure of 23S rRNA (Ban et al., 2000).

Despite the different compositions, both subunits contribute to the binding sites of transfer RNA (tRNA) molecules at the (aminoacyl) A site, the (peptidyl) P site, and the (exit) E site (Figure 1.1) (Steitz, 2008, Korostelev et al., 2008). The A site binds the aminoacyl-tRNA that is about to be combined into the growing polypeptide chain, the P site positions the peptidyl-tRNA and E site is occupied by all deacylated or "uncharged" tRNAs that is ready to exit from the ribosome (Steitz, 2008).Three stages are involved during the protein synthesis; initiation, elongation, and

termination. During the initiation process, a specific tRNA molecule binds to the P site and stays there until the termination stage. The first elongation process takes place when the incoming anticodon from the tRNA is delivered to A site together with its elongation factors. At this point, the 30S subunit ensures the correct pairing between codon of the messenger RNA (mRNA) and the anticodon of the tRNA at A site to be combined to the growing polypeptide chain at P site. Peptidyl transferase at the PTC mediates the process. This whole elongation process is repeated until a stop codon is reached at A site, which leads to the release of newly synthesized polypeptide. The newly synthesized polypeptide goes through PTC that is also part of ribosomal tunnel to exit the ribosome.

PTC is located in the middle of the face of the particle that interacts with the 30S ribosomal subunit and it is responsible for two principal chemical reactions of protein synthesis: peptide bind formation and peptide release (O'Connor et al., 2004, Polacek and Mankin, 2005). This centre is composed entirely of 23S rRNA, Domain V, which is also known as the "peptidyl transferase loop" and catalyzes the formation of peptide bonds during elongation process (O'Connor et al., 2004, Nissen et al., 2000, Vimberg et al., 2004).

There is a tunnel, with ~100Å long and diameter between 10 Å and 20 Å, starts at PTC where the nascent polypeptide passes through (Korostelev et al., 2008, Voss et al., 2006). Apart from Domain V, which makes up the PTC, the narrowest portion of it is formed by the protruding loops of two ribosomal proteins, L4 and L22 peptide chains, which approaches the tunnel from the opposite sides and intercalate between rRNA segments of the 23S rRNA (Korostelev et al., 2008). In order for a nascent

peptide to be translated from ribosome, it must go through a tunnel to exit the ribosome. In the earlier study of the ribosome, tunnel was an exception until the late 1960s when Malkin and Rich, and Blobel and Sabatinin concluded that there was a channel within which the nascent peptides were transferred as they found 30-40 amino acid residues were not catalyzed by proteolytic enzymes (Malkin and Rich, 1967, Sabatini and Blobel, 1970). Frank group further proved an exit tunnel that spanned through the ribosome cryo-electron microscopy in 1995 (Frank et al., 1995).

Another ribosomal proteins contribute to the PTC is L39e as found in the 50S ribosomal subunit of *Haloarcula marismortui (H. marismortui)* (Fulle and Gohlke, 2009). The PTC provides spatial and chemical environment that catalyzes peptide bond formation (Hermann, 2005).

Being the main player in protein synthesis, ribosome has been a target to many clinically relevant antibiotics such as macrolides. The macrolides, in general, bind adjacent to the PTC near to the narrowest portion of the nascent polypeptide exit tunnel (Vimberg et al., 2004). It is reported that the tight binding of the macrolide is achieved through two types of interactions, the hydrophobic and van der Waals with the lactone ring and the RNA-based tunnel surface as well as hydrogen bonding of the macrolide sugar residues to rRNA (Vimberg et al., 2004). Other than direct the interactions ribosome-macrolides, water-mediated contacts to the binding pocket is contributed by ribosomal proteins L3, L4, L22 and L34 (Hermann, 2005). The crystal structures show the macrocyclic binds within the tunnel and the desosamine sugar extended towards the PTC (Hermann, 2005). The various macrolides bind adjacent to the PTC from the crystal structure, about 10–15 Å into the entrance

region of the polypeptide exit tunnel, where they sterically block the passage of the nascent peptide (Hermann, 2005). One evidence from chemical probing and phylogenetic data suggest that the peptidyl transferase loop is folded into a conserved tertiary structure that determines its function in protein synthesis, which process can be blocked by a range of antibiotics that interact with this region (Douthwaite and Aagaard, 1993). The geometry of the PTC cavity, which a small radii provides structural advantages for antibiotic binding in the curved surfaces, bury larger surface areas and can establish additional interactions, thus increases antibiotic affinity (Polacek and Mankin, 2005). Erythromycin, a type of macrolides, binds at the upper portion of exit tunnel and can protrude their appendages into PTC cavity (Polacek and Mankin, 2005).

Apart from that, metal ions also contribute to the structural stability of the large ribosomal subunit. It has been shown for decades that the structure and function of the ribosome are strongly influenced by the presence of metal ions, sodium, potassium, especially magnesium (Klein et al., 2004). Magnesium ions are commonly associated with folding, stability, and catalytic activity of RNA molecules (Auffinger et al., 2004). The negative charge density that is associated with the RNA phosphate backbone can be neutralized by magnesium ions. Magnesium is the most abundant intracellular multivalent cation and has the highest charge density of all biologically available ions, due to its relatively small ionic radius (Klein et al., 2004).

There are two magnesium ions present in the crystal structure of ERYA bound to 50S ribosomal subunit of *D.radiodurans*. The previous study on the molecular interactions of ERYA with the wild type 50S ribosomal subunit showed that magnesium ions are not directly involved in the binding of ERYA but in maintaining the stability of the binding pocket (Wahab et al., 2008).

All the massive understanding of the ribosome structure and its function explained above have been made possible by the electron microscopy and X-ray crystallography, which provides the structure at the atomic resolution of both the large and small subunits. Two groups, Yonath and Wittman and the group at Pushchino began to study crystallization ribosome two decades ago and the first electron density map of the ribosome that showed features of duplex RNA at 9 Å resolution x-ray crystallographic of the large subunit of *H. marismortui* and the atomic resolution has become better and better up to 2.4 Å atomic resolution (Ban et al., 2000). The polypeptide exit tunnel was first observed by Unwin and co-workers in electron microscopy images of the ribosome in the early 1980s (Steitz, 2008, Milligan and Unwin, 1982). The recently determined high-resolution structures of ribosome with over two dozen different antibiotics has paved the way for a detailed examination of specific binding modes, leading to a better understanding of drug selectivity and the principles of drug resistance (Auerbach et al., 2004). All the x-ray crystallography data and electron microscopy images are available at Protein Data Bank (PDB).

1.3 Macrolides

The awareness of drug interactions either as bacteriostatic or bacteriocidal have claimed great responses to develop new clinically active drugs due to resistant mechanisms arises from the previous drugs. The mechanism of actions of these antibiotics is to target the bacteria's protein, deoxyribonucleic acid (DNA), and/or cell wall. One of the macromolecules that have been the drug target's site is the ribosome. Ribosome is an essential organelle in bacteria that is responsible for protein synthesis from amino acid.

The interaction between ribosome and antibiotic either as bactericidal or bacteriostatic at molecular level is an important tool to understand on how the mechanism of protein synthesis inhibited by the drug's action. Studying the interaction at this level is believed to assist the development of better antibiotics and sequentially fight the antibiotics resistance mechanism. One of such antibiotics that target ribosomal subunit in bacteria is macrolides, which reversibly bind to 50S ribosomal subunit. Thus, macrolides are bacteriostatic agents. They interfere with the peptidyl transferase function of the ribosome.

Since the late 1950s, the macrolides class of antimicrobial agents has been broadly used to treat a range of infectious conditions (Rubinstein, 2001). The macrolides are generally acknowledged to be one of the safest antibiotics available to the practising clinicians for the treatment of mild to moderate community-acquired bacterial infections (Abu-Gharbieh et al., 2004). They become primary antibiotics of choice due to their important characteristics; moderately broad spectrum antimicrobial action, an orally effective route of administration, and a relatively high margin of

safety (Parnham and Bruinvels, 2002). They have been particularly useful as treatment for patients who are allergic to penicillin and are effective against pneumococcal, streptococcal and mycoplasma infections, making them clinically effective for the treatment of upper and lower respiratory tract infections (Rubinstein, 2001). These clinically important antibiotics are more active against Gram-positive than Gram-negative bacteria. In Gram-negative bacteria, macrolides are frequently conferred to resistant due their relative impermeability of the cellular outer membrane because of the hydrophobic nature of macrolides themselves (Schlünzen et al., 2003).

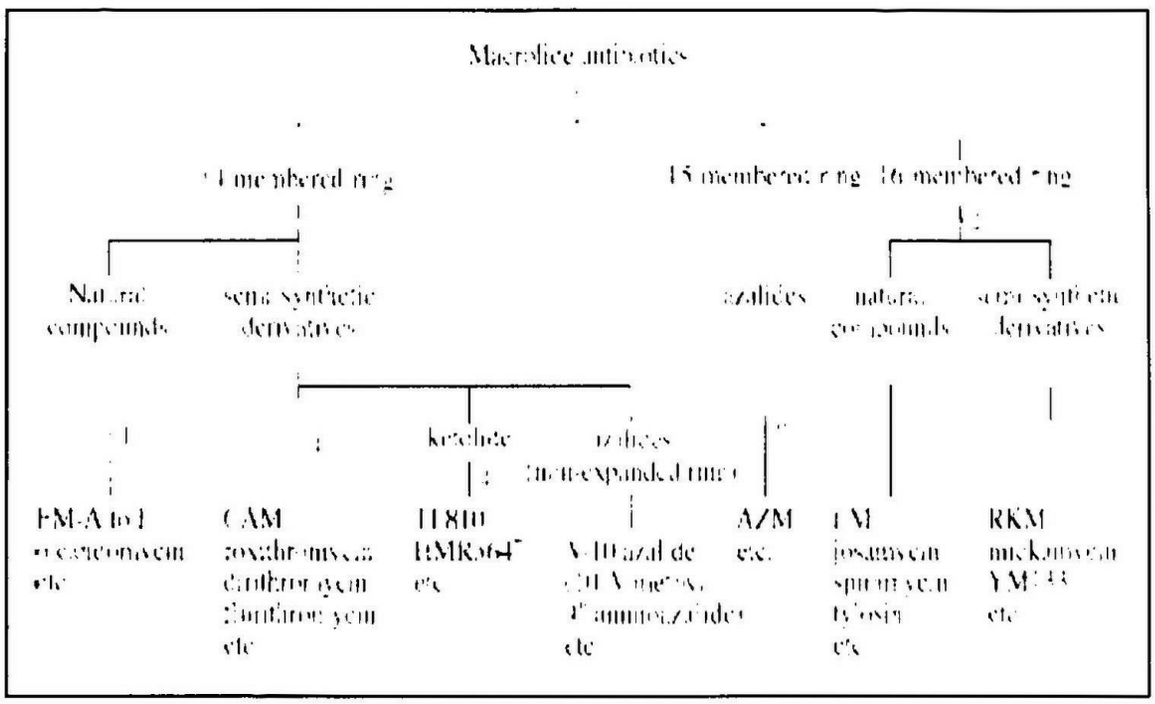


Figure 1.4: Several types of clinically active macrolides. Symbols 1, 2, 3, and 4 indicate first, second, third and fourth generation macrolide antibiotics (Nakajima, 1999).

The macrolides could be identified in various forms, which are monolactone, polyene, spiroacetal compounds, cytochalasins and micropylides, and macrocylic lactones, which are having more than one ester linkage (Omura, 2002). Macrolides with monolactone ring have a variety of rings ranging from 8- to 42- membered lactone rings and can be classified according to the number of carbon atoms in the macrocyclic ring (Rubinstein, 2001, Retsema and Fu, 2001). Macrolides with 14-, 15- and 16-membered lactone rings ornamented with various side chains are clinically the most useful anti-infective (Omura, 2002, Schlünzen et al., 2003, Gaynor and Mankin, 2005, Pfister et al., 2004). Figure 1.4 shows the three groups of clinically active macrolides and their generation. Macrolides producing organisms are not only limited to actinomycetes but also include myxobacteria, fungi, lichens, algae, and plants, although actinomycetes produce the largest number of macrolides, (Omura, 2002).

Most originally isolated from *Streptomyces* species, the macrolides form probably the largest group of known natural products (Rubinstein, 2001). Macrolides are a diverse class of natural products and semisynthetic derivatives based on polyketide macrolactone ring substituted with one or more non-nitrogenous and/or amino acid sugar moieties (Omura, 2002). Physically, macrolide antibiotics are colorless and crystalline substances that are poorly water soluble, and they do dissolve in more organic solvents (Sabri, 2004). These lipid soluble antibiotics are often used in ester forms to enhance oral bioavailability and to improve oral tolerance.

Macrolides contain multiple functional group, thus make them possible to undergo a large number of chemical reactions. Erythromycin, a type of natural macrolides, is the prototype for macrolides for the development of new synthetic macrolides (Rodvold and Piscitelli, 1993). They are also semisynthetic products, which could be divided into three different subgroups; substituent modification, such as roxithromycin and clarithromycin, modifications of aglycone A namely, azithromycin, and modification of the C-3 α-L-cladinose, for example, ketolides (Retsema and Fu, 2001). The sugar moieties that attach to the lactone ring are cladinose and desosamine. Referring to Satoshi Omura in his book, Macrolide Antibiotics, Woodward initially proposed the term macrolides for macrocylic antibiotics (Omura, 2002). The antibiotics activities from this group stem primarily from the presence of macrocylic lactone ring.

Macrolides have always been acknowledged as bacteriostatic, however they can also act as bacteriocidal under certain conditions or against specific microorganisms. Macrolides are considered to be one of the classic bacteriostatic drug classes, but erythromycin, azithromycin, and clarithromycin have shown bactericidal activity *in vitro* against *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Pankey and Sabath, 2004). In exhibiting bacteriostatic activity, they reversibly bind to the 23S rRNA in the 50S ribosomal subunit and inhibit transpeptidation/translocation process. This cascade mechanism is followed by premature detachment of partial peptide chains and cell death (Rubinstein, 2001, Xiong et al., 2005, O'Connor et al., 2004). Macrolides bind to the large ribosomal subunit near the narrow part of the nascent peptide exit tunnel in the ribosome (Xiong et al., 2005, Tenson et al., 2003).

Even there are various types of macrolides that bind to the ribosome but the interaction between the two entities show similar orientation of the lactone ring that share a set of contacts with 23S rRNA (Vimberg et al., 2004). Also, the macrolides-ribosome binding may be toxic for bacterial cells because of the accumulation of peptidyls-tRNA in the cells depletes the pools of free tRNA (Tenson et al., 2003).

Biochemical and genetic data have revealed that the macrolides interact with the loop helix 35 in Domain II of the bacterial 23S rRNA and with the PTC in Domain V (Wahab et al., 2008, Retsema and Fu, 2001). In comparison to the structure, macrolides are small relative to the ribosome and any interactions between them would be possible only if the rRNA is folded so that helix 35 and the peptidyl transferase loop are adjacent. Evidence from other approaches, including phylogenetic comparisons of rRNA sequences and RNA cross-linking, strongly supports the idea of contact between these two rRNA regions (Vester and Douthwaite, 2001). The macrolides-binding pocket is primarily located in Domain V (Pfister et al., 2004). A number of nucleotide residues in domain V of 23S rRNA interact with the macrolides molecule, with important contacts are markedly contributing to the strength of interaction of the macrolides molecules with the ribosome, are formed between the C5 mono- or disaccharide side chains of 14- 15- and 16-membered ring macrolides and rRNA (Gaynor and Mankin, 2005). A macrolide molecule is coordinated in its binding site by multiple hydrophobic and hydrogen bonds, and few with covalent bonds between its functional groups and 23S rRNA and these interactions with RNA accounts for most free energy of the drug binding (Gaynor and Mankin, 2005).

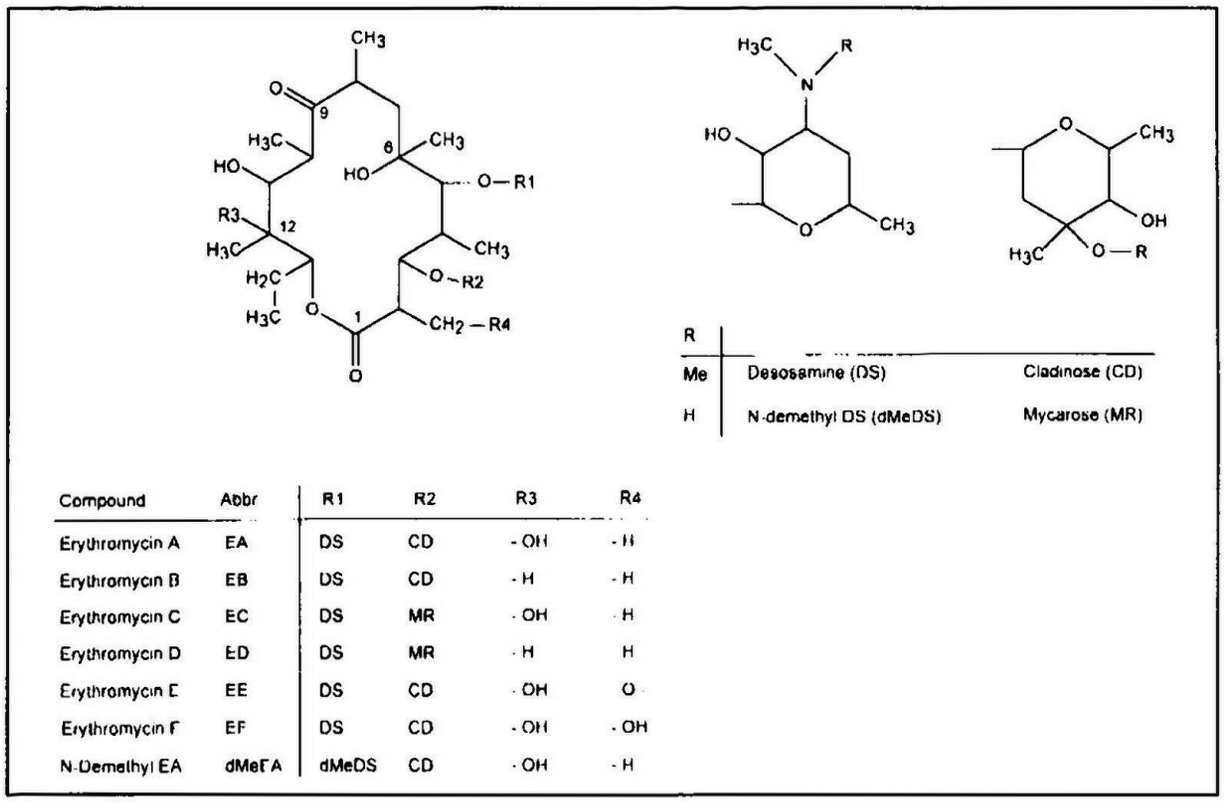
1.4 Erythromycin and Erythromycin A

Erythromycin is a macrolide with a 14-membered ring and a number of its derivatives contain a cladinose sugar residue at the C-3 carbon atom of the lactone ring and a desosamine sugar residue at position C-5. It is also known as polyhydroxylactone (Xiong et al., 2005). It has been reported that in 1952 erythromycin was first obtained from soil bacterium, *Saccharopolyspora erythreus,* from a very complex fermentation (Nakajima, 1999, Wahab et al., 2008). There are four generations of macrolides that are distinguishable based on its improvement and erythromycin is the first generation of macrolide antibiotics contains 14-membered ring (Nakajima, 1999).

In terms of stability, erythromycin is insoluble in water but soluble in alcohol and hydrochloric acid; thermally unstable in solutions containing water; and pH dependent with the optimal pH values found in literatures is between pH 7 and 8 (Brisaertm et al., 1996, Sabri, 2004). The tertiary amine of desosamine sugar confers a basic characteristic to erythromycin (pKa *8.8*) (Kanfer et al., 1998). The antimicrobial activity of erythromycin decreases with increasing incubation temperature exposure at 40, 60, 75, and 100 °C gradually (Sabri, 2004).

Erythromycin is a mixture of naturally occurring macrolides antibiotics produced by *Streptomyces erythreus (S. erythreus).* Erythromycin is a crystalline colorless compound, slightly soluble in water and much more active in an alkaline than an acid medium(Cornell, 2000). Numerous erythromycin related glycosides have been isolated from the fermentation broths of blocked mutants and producing strains of the

erythromycin producing organism *S. erythreus* and only the letter-designated erythromycin A~F have been shown to be primary biosynthetic compounds (Martin et al., 1982). From recent analytical methods, such as liquid-chromatography, these compounds, Erythromycin A through D, are present in commercial samples of erythromycin (Kibwage et al., 1985). All structures of erythromycin A through F (Figure 1.5) hold similar structures except at three different positions with different substituent, namely R at C12 and R' at C2 of the lactone ring and R" at C3 of the cladinose sugar. This substituent could either be methyl group, hydrogen atom or hydroxyl group.



Compound	Abbr	R1	R2	R3	R4
Erythromycin A	EA	DS	CD	-OH	-H
Erythromycin B	EB	DS	CD	-H	-H
Erythromycin C	EC	DS	MR	-OH	-H
Erythromycin D	ED	DS	MR	-H	H
Erythromycin E	EE	DS	CD	-OH	O
Erythromycin F	EF	DS	CD	-OH	-OH
N-Demethyl EA	dMeEA	dMeDS	CD	-OH	-H

Figure 1.5: The chemical structure of Erythromycin A through F and their differences at selected substituent (Kanfer et al., 1998).

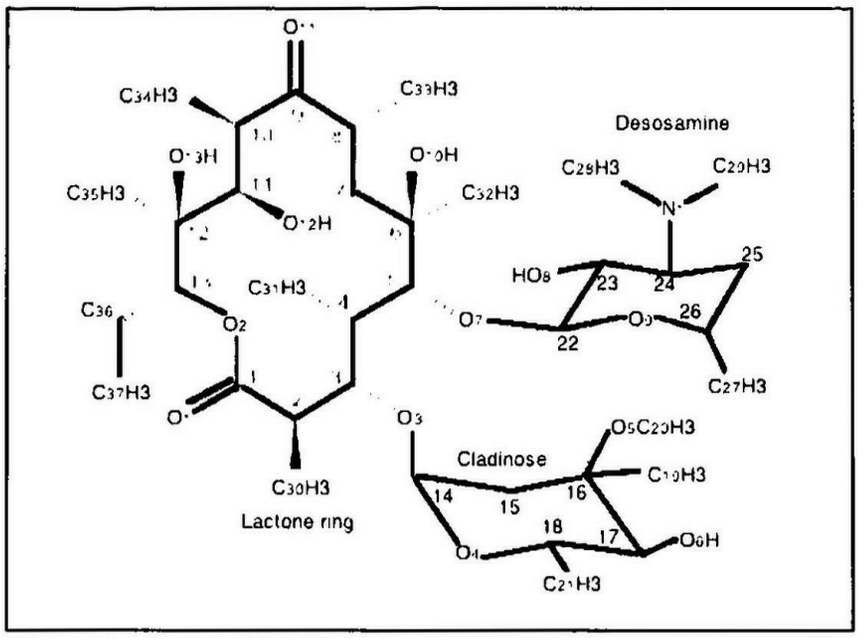


Figure 1.6: The structure of ERYA showing its lactone ring and the two sugar moieties, desosamine and cladinose (Pfister et al., 2004).

Erythromycin A (ERYA) (Figure 1.6) is the most active antimicrobial agent while erythromycin B to F are the derivatives product of the fermentation. The determination of minimum inhibitory concentration (MIC) values of erythromycin A, B, C and D against 21 gram-positive and 15 gram-negative microorganisms show that erythromycin B is somewhat less active than ERYA, and erythromycin C and D show about half that activity or even less (Kibwage et al., 1985). ERYA shows high specificity and affinity for the bacterial 50S ribosomal subunit with dissociation constant, (K_d) ~ 10^{-8} M (Wahab et al., 2008, Pestka, 1974).

ERYA binds at the top of the peptide exit tunnel in the 50S ribosomal subunit and halts protein synthesis by blocking the nascent peptide's access to the tunnel (O'Connor et al., 2004, Ishida and Hayward, 2008). In the experimental approach, it has been shown that erythromycin bind 1:1 stoichiometry to the 50S ribosomal subunit of *E. coli* (Usary and Champney, 2001).

1.5 Erythromycin Resistance

Antibiotic resistance occurs when an antibiotic has lost its ability to effectively control or kill bacterial growth and continue to multiply to cause more harm. The molecular mechanisms by which bacteria become resistant are manifold. The most common mechanisms of resistance are excretion of the drug from the cell, drug inactivation, and modification of the drug target site (Gaynor and Mankin, 2005, Vester and Douthwaite, 2001, Pfister et al., 2004). Erythromycin is bound to resistance just like other antibiotics have been demonstrating. Modifications of the drug target site can either be methylation or base substitutions on selected nucleotides. These modifications occur on the large ribosomal subunit, 50S, specifically on 23S rRNA. The inhibitory characteristics of each of these drugs are presumably determined by a combination of the ability of the drugs to gain access to its target site of the ribosome, its binding affinity at the site and its effect on perturbing or blocking access to the rRNA structure (Hansen et al., 1999).

Mutations occur in protein L4, L22, and other chains of the ribosome as well as few positions of nucleotides namely 750EC, 2058EC, 2059EC, and 2069EC (O'Connor et al., 2004, Tu et al., 2005). These mutations have been found to confer macrolides resistance. A2041 of *D.radiodurans* is one of the nucleotides of the peptidyl transferase loop that is not conserved among all phylogenetic domains (Schlünzen et al., 2001). Base substitution at 2041 from A to G explains the selectivity of macrolides to resistance because mitochondrial and cytoplasmic rRNAs of higher eukaryotes have guanine at the respective position (Schlünzen et al., 2001, Vester and Douthwaite, 2001, Auerbach et al., 2004). A2058EC to G is the first rRNA mutation shown to confer erythromycin resistance and is presently the most frequent

clinically isolated substitution, as well as it gives the highest level of resistance to 14-membered macrolides (Vester and Douthwaite, 2001).

In the wild type, the drug's position in the binding pocket involves hydrogen bond interactions of the amino sugar at C5 of the lactone ring with the A2058EC base, and possibly with the A2059EC base and additional interactions between the hydrophobic edge of the lactone ring and the minor groove surface of A2058EC and A2059EC, which are also important for macrolide binding (Pfister et al., 2004). Resistance happens as the nucleotides whose bases are engaged in hydrogen bonding are mutated (Polacek and Mankin, 2005). It has also been reported that the increased size at this position, from A (33 atoms) to G (34 atoms), eliminates interactions with the desosamine sugar (Auerbach et al., 2004). Another report shows that mutations at position A2057EC and A2058EC (A → G, or →U) all of which confer drug resistance, induce a more open conformation in the peptidyl transferase loop, and the affinity of the drug interaction is reduced 20 fold in the A2057EC mutant, 10^3- fold in the U2058EC mutant and 10^4-fold in the G2058EC mutant(Douthwaite and Aagaard, 1993). A single base substitution is introduced at 23S rRNA nucleotides A2058EC to C, G or U and the MIC values for the mutant rRNA strains are determined for 14-, 15-, and 16-membered macrolides. The results indicate that the MIC values for ERYA with a single base substitution at A2058EC increases to more than 1024 μg/mL from only 16 μg/mL in the wild type (Pfister et al., 2004). In short, either single or multiple base substitution can alter the appearance of the drug target site, so that it is no longer effectively recognized or bound by the drug (Stephen Douthwaite, 1993).

1.6 Historical Perspective on Biological Macromolecules Simulations

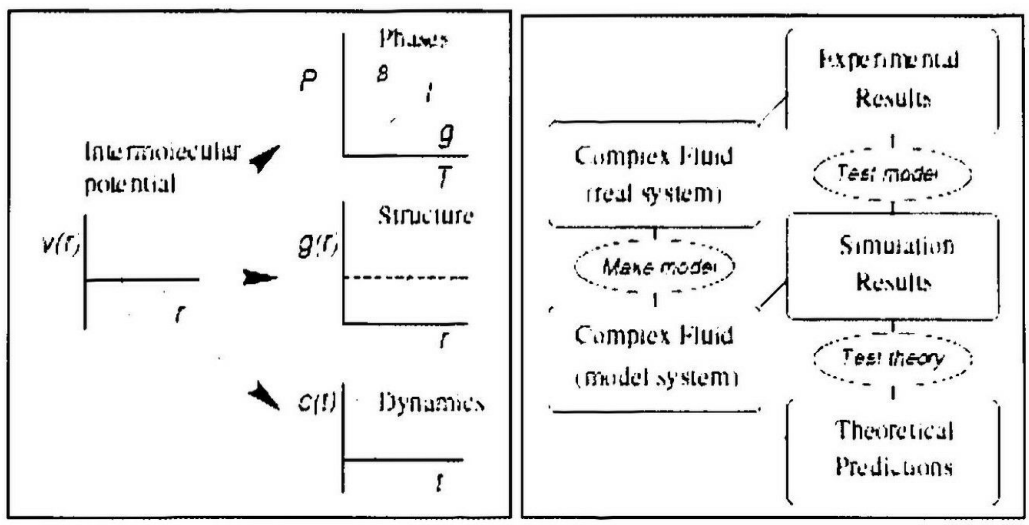


Figure 1.7: Simulations as a bridge between microscopic and macroscopic by theory and experiment (Allen, 2004).

Simulations can act as a bridge to correlate the macroscopic properties of a system through microscopic length and time scales to obtain 'exact' predictions of bulk properties (Figure 1.7) (Allen, 2004). Due to this, simulation has been a great tool to study complex biological macromolecules so that they could provide ultimate detail of the molecular features that involved in the conformational changes, protein folding, protein stabilities, ions transport in biological system and molecular recognition.

The first biological macromolecules simulations using protein as a subject matter dated back in 1977 (Loccisano, 2007, McCammon et al., 1977). The concern was on the bovine pancreatic trypsin inhibitor (BPTI), a small system with 58 residues or 885 atoms, which lasted 9.2 picosecond (ps) performed in vacuum. This simulation had changed the conventional view that protein was a rigid structure but it could

exhibit substantial fluctuations (Karplus, 2002, Loccisano, 2007, Hansson et al., 2002). Since then, many studies related to biological macromolecules have been published. Current literatures on molecular dynamics simulations have expanded to solvated proteins, protein-DNA complexes, and lipid systems. The time scales of a simulation have advanced to microsecond with modern computers so that it is possible to study the phenomena of biological interest in real time (Karplus, 2002). The number of atoms in one system has also reached millions, for example, in 2005; researchers at Los Alamos National Laboratory, New Mexico simulated 2.64 million atoms on bacterial ribosome (Sanbonmatsu et al., 2005).

One of the earliest publications related to the molecular dynamics simulations of bacterial ribosome could historically be tracked in the year 1985, by Åqvist and his group (Åqvist et al., 1985). His group studied on the C-terminal fragment of the L7/12 ribosomal protein secondary structure motion and the simulations lasted for 150 ps. The MD results showed good agreement with high-resolution X-ray data in terms of atomic positions, distances and positional fluctuations. From this onwards, the focus of the study has extended not only to the flexibility of ribosome to fluctuate but also interactions of antibiotic with the ribosomal to act as protein synthesis inhibitor. These studies have also expanded to a complete ribosomal structure rather than fragments of the ribosome, when the first complete X-ray structures of the 50S ribosomal subunit at 3.5 Å resolution, was made available in 1980 by Yonath *et al.* (Yonath et al., 1980).

However, due to a very large number of atoms and limitations on computational resources, only in 2005, MD simulation of 70S ribosome for half microseconds was successfully done by Trylska *et al.* using coarse-grained method, by reducing representations for a system with large number of atoms (Trylska et al., 2005, Wahab et al., 2008). The first all atom simulation involving 50S ribosomal subunit and the antibiotic, ERYA was performed by our group in 2008 (Wahab et al., 2008).

1.7 Introduction to Molecular Dynamics

MD, which is a part of computer simulations, is a theoretical study of biological molecules by generating information at the microscopic levels that analyze atoms positions and velocities with respect to times. MD enables us to gain insight into situations that are impossible to study experimentally as the microscopic values will be converted into macroscopic observables such as energy, heat capacities, and pressure. These macroscopic observables are converted via statistical mechanics (Gunsteren and Berendsen, 1990). The statistical mechanics provides demanding mathematical equations that correlate the macroscopic properties with the coordinates and motions of atoms and molecules in the N-body system. MD comes into play by providing means to solve the equation of motion of the particles and evaluate the mathematical formulas. In short, MD simulations enable us to study both thermodynamic properties and/or time dependent (kinetic) phenomenon.

MD produces successive trajectories of the system by integrating Newton's law of motions. There are three Newton's law of motions; the first law, a body continues to move in a straight line at constant velocities unless a force acts upon it, the second law, force equals the rate of change of momentum, and the third law, to every action there is an equal and opposite reaction (Leach, 2001). MD solves the classical equation of motion, the Newton's second law (in the formula below) on each atom in the system being investigated.

$$F_i = m_i \cdot a_i$$

F is the force exerted on the particle, m is its mass and a is its acceleration. The acceleration of each atom could be calculated in the system. Trajectories are produced from the application of the equation that describes the positions, velocities and accelerations of the particles as they vary with time. From this trajectory, the average values of properties can be determined. MD is a deterministic method whereby the state of the system at any future time can be predicted by its current state (Leach, 2001). This can be computed by combining the forces of each atom with its current position and velocity to generate the new position and velocity that differs in time with force to be constant on each atom during the time interval.

There are few widely used programs to run MD such as CHARMM (Books et al., 2004), GROMACS (Spoel et al., 2005), and AMBER (Pearlman et al., 1995). The parameters to run MD simulations are completely under the control of the user to determine the given properties to be examined. However, MD is a theoretical estimate thus; MD still uses experimental results to validate its methodology by comparing the simulations and experimental results as error are made during the calculations on each MD simulations are impossible to be quantified (Karplus and McCammon, 2002). Moreover, the reliability of predictions made on the basis of MD simulations will be basically depend on two factors; whether the molecular model and force field used are adequately accurate and whether the configuration space accessible to the molecular system with the low-energy configurations has been thoroughly searched (Gunsteren and Berendsen, 1990). Thus, before running MD simulations, user will need to determine the length of simulations, pressure and