Title
Structural Studies of the Elongation Cycle of Protein Synthesis and Its Inhibition by Antibiotics

Permalink
https://escholarship.org/uc/item/49m7m9bs

Author
Dunkle, Jack A.

Publication Date
2010

Peer reviewed|Thesis/dissertation
Structural Studies of the Elongation Cycle of Protein Synthesis and Its Inhibition

by Antibiotics

By

Jack Albert Dunkle

A dissertation submitted in partial satisfaction of the requirements for the degree

of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jamie H. D. Cate, Chair
Professor James M. Berger
Professor Susan Marqusee
Professor David E. Wemmer

Fall 2010
Abstract

Structural Studies of the Elongation Cycle of Protein Synthesis and its Inhibition by Antibiotics

by

Jack Albert Dunkle

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Jamie H. D. Cate, Chair

Protein synthesis takes place in four stages: initiation, elongation, termination and recycling. Elongation consists of delivery of a charged tRNA to the ribosome, peptide bond formation and translocation of the mRNA and tRNA, three steps forming a cyclic process, which is repeated for every amino acid added to a growing polypeptide chain. The result of the elongation cycle is the translation of the triplet genetic code contained in an mRNA, into the amino acid sequence of a protein. Since the 1960s it was appreciated that complex conformational changes must occur on the ribosome to accomplish translocation of mRNA and tRNA. Here I report x-ray crystallographic studies which shed light on how mRNA and tRNA are manipulated by the ribosome and mechanisms used by certain antibiotics to inhibit the elongation cycle of protein synthesis.

Specifically research reported here argue that tRNA translocation is a stepwise process that involves discrete structural intermediates of the ribosome. I report structural evidence that the antibiotics clindamycin and chloramphenicol inhibit protein synthesis by interfering with aminoacyl-tRNA positioning in the peptidyl transferase center. Also, I hypothesize based on structural data and phylogenetic analysis, that the identity of the ribosomal RNA residues numbered in E. coli 752, 2055 and 2609 contribute to the specificity of many antibiotics for binding to bacterial, rather than archaeal or eukaryotic ribosomes.
Dedication

This dissertation is dedicated to my parents, David Dunkle and Judith Dunkle, who encouraged and assisted me in the formative early years of my education, who knew the importance of schoolwork, but also the importance of my diverse extracurricular interests. This dissertation is dedicated also to Dr. Charles Turnbough, who encouraged me to pursue a career in science, and my wife, Sandra Nuwayhid, who tolerated my absence during the many nights and weekends required to complete the research described in the following pages.
# Contents

List of Figures...........................................................................................................vi
List of Tables...............................................................................................................viii

## 1. Introduction to Protein Synthesis

1.1 The Components of the Ribosome.................................................................1
1.2 The Activity of the Ribosome........................................................................1

## 2. Structures of the Ribosome in an Intermediate Ratcheted Conformation
Reveal that mRNA and tRNA Translocation Occurs Through Discrete Steps

2.1 Abstract ........................................................................................................5
2.2 Introduction to mRNA and tRNA translocation on the Ribosome..............5
2.3 Results and Discussion
   2.3.1 Structural Characterization of a New Intermediate Ratcheted
         Conformation of the Ribosome.............................................................6
   2.3.2 Interactions Between the Anticodon Stem Loop of tRNA and the 30S
         Subunit Control tRNA Positioning.........................................................8
   2.3.3 Structural and Single Molecule Data Suggest Translocation
         Proceeds Through Discrete Intermediates.............................................9
2.4 Materials and Methods
   2.4.1 Ribosome Purification and Crystallization...........................................10
   2.4.2 Diffraction Data Collection...................................................................10
   2.4.3 Refinement and Model Building.........................................................10
   2.4.4 Superpositions and Rotation Calculations.........................................11

## 3. Structures of the E. coli Ribosome with Antibiotics Bound Near the
Peptidyl Transferase Center Explain Spectra of Drug Action

3.1 Abstract.........................................................................................................12
3.2 Introduction to Mechanistic Studies of Protein Synthesis Inhibition..........12
3.3 Results and Discussion
   3.3.1 The Structure of Erythromycin Bound to the E. coli Ribosome is
         Similar to the Structure of the Compound Bound to the H. marismortui
         Ribosomal Subunit..................................................................................14
3.3.2 The Structure of Telithromycin Bound to the *E. coli* Ribosome Reveals a New Interaction with rRNA Explaining Resistance Mutations...

3.3.3 The Structure of Clindamycin Bound to the *E. coli* Ribosome Reveals that the Compound Inhibits A-site tRNA Positioning...

3.3.4 The Structure of Chloramphenicol Bound to the *E. coli* Ribosome Reveals Its Binding Site on the Eubacterial Ribosome...

3.3.5 Several Divergent Nucleotides Surrounding the Peptidyl Transferase Center Can Determine the Species Specificity of Protein Synthesis Inhibitors...

3.4 Materials and Methods

3.4.1 Introduction of the Antibiotics into the Crystals...

3.4.2 Diffraction Data Collection, Refinement and Model Building...

3.4.3 rRNA Sequence Alignments...

3.4.4 Chemical Probing Experiments...

4. Structure of the *E. coli* Ribosome Bound to the New Ketolide CEM-101

4.1 Abstract...

4.2 Introduction to Ketolide Antibiotics...

4.3 Results and Discussion

4.3.1 Competition binding experiments show CEM-101 has the same binding site as previous ketolides...

4.3.2 The Alkyl Aryl Heterocycle of CEM-101 Has More Stable Interactions with rRNA Than Telithromycin...

4.3.1 Any Modifications Larger Than a Fluorine at the C2 Position Will Lead to Steric Clashes with rRNA...

4.4 Materials and Methods

4.4.1 Introduction of the Antibiotics into the Crystals...

4.4.2 Diffraction Data Collection, Refinement and Model Building...

4.4.3 Competition experiments...

5. Summary and Conclusions
5.1 Conclusions Regarding Structures of the Ribosome in Intermediate States of Ratcheting..................................................................................................................26
5.2 Conclusions Regarding Structures of the Ribosome with Antibiotics Bound in or near the Peptidyl Transferase Center.................................................................27
5.3 Future Prospects and Challenges for Mechanistic Studies of Protein Synthesis.................................................................................................................................28

Citations..................................................................................................................30

Figures
Chapter 1 Figures....................................................................................................44
Chapter 2 Figures...................................................................................................53
Chapter 3 Figures...................................................................................................57
Chapter 4 Figures...................................................................................................63
Chapter 5 Figures...................................................................................................66

Tables
Chapter 2 Table.....................................................................................................68
Chapter 3 Tables...................................................................................................69
Chapter 4 Tables....................................................................................................71
List of Figures

1-1 Major Features of the Structure of the \textit{E. coli} Ribosome..............................................44
1-2 Binding Sites for tRNA in the Ribosome..........................................................45
1-3 Structural Features of the 30S Ribosomal Subunit..............................................46
1-4 Conformational changes in the 30S subunit associated with translocation....47
1-5 The GTPase Center of the 50S Subunit..............................................................48
1-6 Dynamics of the L1 Arm....................................................................................49
1-7 Path of mRNA into and out of the Ribosome.......................................................50
1-8 Interactions of Acceptor Ends of tRNAs with 23S rRNA in the 50S..............51
1-9 Example Images of Ribosome Crystals and X-ray Diffraction.........................52
2-1 Rotated States of the Ribosome...........................................................................53
2-2 Structure of the Apo-70S Ribosome in an Intermediate State of Rotation.....54
2-3 Contacts Between the Ribosomal Subunits in Apo-70S and R2 State.............55
2-4 Changes in the Position of the Head Domain in the 30S State R2.................56
3-1 Binding Sites for Antibiotics in the PTC and Peptide Exit Tunnel.............57
3-2 Erythromycin Bound to the \textit{E. coli} Ribosome......................................................58
3-3 Telithromycin Bound to the \textit{E. coli} Ribosome.....................................................59
3-4 Footprinting Experiments Show Telithromycin Protects A752 in \textit{E. coli} and \textit{S. aureus}...........................................................................................................60
3-5 Clindamycin Bound to the \textit{E. coli} Ribosome......................................................61
3-6 Chloramphenicol Bound to the \textit{E. coli} Ribosome..............................................62
4-1 The Chemical Structures of Erythromycin, a Macrolide, and the Ketolides
Telithromycin and CEM-101..........................................................63
4-2 Binding of CEM-101 to Wild Type Bacterial Ribosomes......................64
4-3 The Structure of the *E. coli* Ribosome Bound to CEM-101..................65
5-1 Large Scale Motions in the Ribosome During Translocation...............66
5-2 Intermediate States of Subunit Ratcheting....................................67
List of Tables

2-1 Diffraction and Refinement Statistics for Crystals of Ribosomes.................68

3-1 Conservation of Peptidyl Transferase Center And Adjacent Peptide Exit Tunnel Nucleotides........................................................................................................69

3-2 Diffraction and Refinement Statistics for 70S Ribosome Complexes with Antibiotics........................................................................................................70

4-1 Dissociation Constants (K_D) of CEM-101 and Other Macrolides Binding to E. coli or S. aureus Ribosomes................................................................................71

4-2 Diffraction Statistics for Crystals of the E. coli 70S Ribosome Complexed with CEM-101........................................................................................................72
1. An Introduction to Protein Synthesis and the Ribosome

1.1 The Components of the Ribosome

Partially taken from Dunkle, J.A. and Jamie H.D. Cate. An Introduction to the Structure and Function of the Ribosome, EcoSal, in revision.

The ribosome in E. coli is a 2.4 MDa ribonucleoprotein complex composed of 58 proteins and three ribosomal RNAs (rRNA). These proteins and rRNAs assemble into two particles of different sizes, the small ribosomal (30S) subunit and the large ribosomal (50S) subunit. The 30S and 50S subunits interact to form the intact 70S ribosome. Proteins in the 30S subunit are named S1-S21, “S” for small ribosomal subunit, and proteins in the 50S subunit are named L1-L36, “L” standing for the large ribosomal subunit. The protein numbering scheme is based on the electrophoretic mobility of the proteins (Kurland, 1972), and a comprehensive phylogeny of these proteins can be found on the web at the ExPASy public database (http://www.expasy.org/cgi-bin/lists?ribosomp.txt). The small ribosomal subunit is assembled around 16S rRNA, a 1542 nucleotide (nt) RNA in E. coli, whereas the large subunit assembles around the 5S and 23S rRNAs, 120 nts and 2904 nts, respectively. As with the ribosomal proteins, the genomic era has provided a wealth of phylogenetic information with which to derive structural and functional insights into the ribosome. A particularly useful resource for ribosomal RNAs has been compiled by Robin Gutell at the Comparative RNA Website (http://www.rna.ccbb.utexas.edu/).

1.2 The Activity of the Ribosome

Protein synthesis is a multi-step process, and the structural features of the ribosome along with the large number of co-factors reflect the complexity of translation. Numerous protein factors in addition to the ribosome contribute to translation in bacteria during the steps of initiation, elongation, termination and recycling, all of which must occur in order to successfully make a complete protein (Allen and Frank, 2007; Berk and Cate, 2007; Petry et al., 2008). These protein factors make intimate contacts to key regions of the ribosome, and will be discussed below in light of our present understanding of the structure and function of the ribosome (Figure 1-1).

The intact ribosome contains three binding sites for substrate tRNAs, termed the aminoacyl-tRNA site (A site), peptidyl-tRNA site (P site), and exit-tRNA site (E site). These three binding sites span the interface between the 30S and 50S subunits (Figure 1-2). On the 30S subunit, the anticodon segment of tRNAs bind in the A and P sites to base pair with codons in the mRNA. The anticodon of E-site tRNA also binds to the 30S subunit, but its interactions with mRNA are not well understood functionally (Selmer et al., 2006). The acceptor ends of tRNAs interact with the large subunit in all three tRNA binding sites, while the elbow of tRNA interacts with elements of the large subunit to help position the tRNA in the correct binding site. Other tRNA binding sites have been identified in translation steps related to translation initiation, mRNA decoding (Valle et al.,
and mRNA and tRNA translocation from one binding site to the next after each peptide bond is formed (Valle et al., 2003b). However, tRNAs have only been seen in states where at least one end of the tRNA is bound in the A, P, or E sites.

The small ribosomal subunit is responsible for decoding the genetic code in mRNA, and is built in a way that allows it to maintain the mRNA reading frame while maintaining a grip on its substrate tRNAs as they are moved from one binding site to the next. Its architecture reflects the conformational dynamics necessary to carry out these diverse functions. The 30S subunit has anthropomorphic features, i.e. “head”, “body”, “platform” (Figure 1-1A, Figure 1-3A), that form a cleft within which mRNA and tRNA anticodons bind. In mRNA decoding, the P site contains a peptidyl-tRNA (or formylmethionyl-tRNA\(^{\text{fMet}}\) after initiation), and the A site is in an “open” configuration. The shoulder of the 30S subunit closes around the A-site tRNA, which is thought to help drive the forward kinetic steps of the aminoacyl-tRNA selection process (Rodnina et al., 2005). The means by which the shoulder of the 30S subunit closes results from the ability of three universally conserved nucleotides in 16S rRNA to read out the correct pairing of the mRNA codon in the A site with the anticodon of the incoming aminoacyl-tRNA. Nucleotides A1492, A1493, and G530 probe the minor groove of the codon/anticodon three base-pair helix to discriminate correct pairing from non-cognate pairing (Figure 1-3B). Correct codon/anticodon pairing is communicated through the ribosome and aminoacyl-tRNA to the GTPase center of the large subunit, accelerating GTP hydrolysis by elongation factor EF-Tu (Figure 1-3C) (Rodnina et al., 2005). The GTPase center will be discussed in more detail below. After the correct aminoacyl-tRNA is accommodated in the A site and subsequent formation of a peptide bond, the P-site tRNA is deacylated and the A-site tRNA is covalently attached to the growing polypeptide. The chemistry of peptide bond formation is now fairly well established from both kinetic and structural studies (Steitz, 2008).

To repeat the elongation process, the mRNA and tRNAs must then translocate by one binding site. The small subunit rearranges in a number of ways to carry out translocation, beginning with a rotation of the small subunit relative to the 50S subunit by about 7°, proposed to be part of a “ratcheting” mechanism (Frank and Agrawal, 2000) (Figure 1-4). Rotation of the small subunit is coupled to movement of tRNAs on the large subunit while maintaining their binding sites on the small subunit, such that the tRNAs occupy “hybrid states” (Moazed and Noller, 1989b; Valle et al., 2003b). These hybrid states of binding have been termed A/P and P/E to reflect the 30S subunit and 50S subunit positioning of the tRNA anticodons and acceptor stems, respectively (Figure 1-4B). Using this nomenclature the pre-translocation orientations of tRNA are A/A and P/P. Next, the head of the small subunit is thought to move in a swiveling and opening motion to allow the tRNAs to pass to adjacent sites on the small subunit (Figure 1-4C). Once the tRNAs have moved, the steps need to reverse to reestablish a starting configuration ready for the next round of mRNA decoding and peptide bond formation. Proteins S12, which interacts with EF-G during translocation (Connell et al., 2007), and S13, which interacts with the 50S
subunit (Schuwirth et al., 2005), have also been shown to play important roles in translocation (Cukras and Green, 2005). However, the precise nature of the conformational changes in the 30S subunit, and their kinetics, remain to be worked out.

Messenger RNA decoding and translocation require several events to occur on the 50S subunit in coordination with the changes that occur in the 30S subunit. First, a complex of four proteins termed the L7/L12 proteins helps to recruit elongation factors to the ribosome, and also help to stimulate their GTPase activity (Diaconu et al., 2005; Mohr et al., 2002). These proteins are quite flexible, which may aid their ability to sample the solution volume around the ribosome to “sweep” elongation factors into the correct binding site on the ribosome (Figure 1-1B, Figure 1-5). Second, an RNA-protein lobe on the large subunit that includes protein L11, termed the GTPase center, moves in and out of the interface region, and also aids in positioning the GTPases involved in protein synthesis (Figure 1-3C, Figure 1-5) (Connell et al., 2007). An RNA hairpin loop that is conserved in all organisms termed the Sarcin-Ricin Loop (SRL) also helps to stimulate the GTPases (Figure 5) (Blanchard et al., 2004). The SRL is the target of a number of toxins that inactivate translation (Yang et al., 2001). Third, an RNA “bridge” from the 50S subunit to the head of the 30S subunit near the A site has been termed the A-site Finger (Figure 1-2C). This RNA helix forms contacts to proteins S13 and S19, depending on the step of translation, and controls the rate and accuracy of translocation (Komoda et al., 2006). Fourth, the central protuberance of the 50S subunit forms key contacts with the elbow region of P-site tRNA that likely helps maintain its position in the P site (Selmer et al., 2006). Finally, the L1 arm of the ribosome, an RNA-protein lobe on the E-site side of the 50S subunit, is quite flexible, and moves in and out of the interface region of the ribosome (Figure 1-6). The L1 region is thought to contribute to the mechanism of translocation by interacting with the elbow of tRNA as it enters the P/E hybrid binding state, and when tRNA is in the E site (Korostelev et al., 2008b; Selmer et al., 2006).

With all of the large-scale rearrangements that occur in the ribosome during translation, it’s a wonder that the 70S ribosome can hold together. The available cryo-EM and x-ray crystal structures reveal the contacts, or “bridges” that occur between the two ribosomal subunits (Schuwirth et al., 2005). The central bridges between the two subunits involve ribosomal RNA only, whereas peripheral bridges include RNA-RNA contacts and RNA-protein contacts (Figure 1-1). An atomic-resolution view of the bridges in ribosomes in a “classical” conformation, i.e. that compatible with tRNAs in the A/A, P/P, and E/E binding sites is available from a number of x-ray crystal structures (Schuwirth et al., 2005; Selmer et al., 2006). However, how the contacts rearrange in the “ratcheted” states required for a number of steps of translation, including initiation, translocation, termination and ribosome recycling is now only known from lower-resolution cryoEM images (Connell et al., 2007; Valle et al., 2003b). Crystal structures of intermediates along the ratcheting pathway, which will be presented in the following pages, reveal that these contacts likely rearrange in a
step-wise manner, beginning at the periphery, and finishing at the center of the interface (Zhang et al., 2009).

The path of mRNA through the ribosome involves a “U-turn” around the neck of the 30S subunit and traverses an entry channel that requires the mRNA to be unfolded (Yusupova et al., 2001). The mRNA extending in the 5’ direction from the P site passes through the E site and exits towards the solvent side of the 30S subunit where it can potentially interact with the 3’ end of 16S rRNA. During translation initiation, an RNA helix can form between the 5’ region of mRNA and the 3’ end of 16S rRNA to form the “Shine-Dalgarno” helix (Figure 7A) (Allen et al., 2005). This helix, along with ribosomal protein S1, helps to regulate translation initiation from most cellular mRNAs. The mRNA extending in the 3’ direction from the P and A sites passes through a channel formed by 16S rRNA and three ribosomal proteins, S3, S4 and S5 (Figure 1-7B) (Yusupova et al., 2001). These three proteins are thought to contribute to an intrinsic helicase activity of the ribosome (Takyar et al., 2005), where the head of the small subunit “shears” above the shoulder domain, moving protein S3 relative to proteins S4 and S5 (Borovinskaya et al., 2007).

The central activity of the ribosome is catalysis of peptide bond formation. The region of the ribosome responsible for catalyzing the reaction is called the peptidyl transferase center (PTC). Structures of the 50S subunit were the first to show without equivocation that the PTC is composed entirely of RNA (Nissen et al., 2000; Schmeing et al., 2005). In other words, the ribosome is essentially a ribozyme. More recent structures of the 70S ribosome reveal that ribosomal proteins L16 and L27 (Voorhees et al., 2009), may serve an accessory role by aiding in tRNA binding and positioning. The growing polypeptide exits the ribosome through a tunnel that is over 100 Å long, and wide enough for protein secondary structure to form (Ban et al., 2000). The surface of the exit tunnel is composed of both RNA and ribosomal proteins. Although the exit tunnel is over 100 Å long, it is not entirely inert. There are pathways of communication from regions within the exit tunnel to the surface of the ribosome where the protein emerges, although the structural basis for how the pathways work is not clear (Beringer, 2008).

In addition to contacts to the elbow region of the tRNA substrates, the 50S subunit also forms base pairs between 23S rRNA the 3’ ends of both P-site and A-site tRNAs, which helps position them within the peptidyl transferase center (PTC) (Figure 1-8A) (Selmer et al., 2006). In the E site, which plays a critical role in helping the tRNA translocation mechanism, 23S rRNA elements form a pocket that binds the terminal adenosine of E-site tRNA and P/E tRNA (Figure 1-8B) (Selmer et al., 2006). Modifications of the 3’ end of tRNA dramatically perturb translocation (Lill et al., 1989). Consistent with biochemical data, the pocket formed by the rRNA is only capable of accommodating a deacylated tRNA.

In conclusion, the preceding discussion makes it clear a great deal of information concerning the structure of the ribosome, its interactions with its substrates and some mechanisms related to catalysis are known. Two areas where questions still linger will be addressed in the following pages, the structural
re-organization of the ribosome during ratcheting and tRNA translocation, and
the inhibition of peptide chain elongation by antibiotics binding in and near the
peptidyl transferase center. With regard to ratcheting and tRNA translocation,
data from new x-ray crystal structures which reveal the details of how the
intersubunit bridges can re-orient themselves during this process, will be
presented. With regard to the inhibition of peptide chain elongation, structures of
the \textit{E. coli} ribosome bound to four antibiotics will be presented which provide the
highest quality data to date for these compounds in the context of the ribosome
of a medically relevant bacterium. Lastly, the structure of an experimental new
ketolide antibiotic, CEM-101, bound to the \textit{E. coli} ribosome will be presented,
which demonstrates that ribosome crystal structures can be of assistance to
medicinal chemists crafting new protein synthesis inhibitors.

2. Structures of the Ribosome in an Intermediate Ratcheted Conformation
Reveal that mRNA and tRNA Translocation Occurs Through Discrete Steps

Partially taken from Zhang, W., Dunkle, J.A. and J.H.D. Cate (2009). \textit{Science}
325, 1014-1017.

2.1 Abstract
Protein biosynthesis on the ribosome requires repeated cycles of ratcheting,
which couples rotation of the two ribosomal subunits with respect to each other,
and swiveling of the head domain of the small subunit. However, the molecular
basis for how the two ribosomal subunits rearrange contacts with each other
during ratcheting while remaining stably associated is not known. Here, we
describe x-ray crystal structures of the intact \textit{Escherichia coli} ribosome, either in
the apo-form (3.5 Ångstrom resolution) or with one (4.0 Ångstrom resolution) or
two (4.0 Ångstrom resolution) anticodon stem-loop tRNA mimics bound, that
reveal intermediate states of intersubunit rotation. In the structures, the interface
between the small and large ribosomal subunits rearranges in discrete steps
along the ratcheting pathway. Positioning of the head domain of the small subunit
is controlled by interactions with the large subunit and with the tRNA bound in the
peptidyl-tRNA site. The intermediates observed here provide insight into how
tRNAs move into the hybrid state of binding that precedes the final steps of
mRNA and tRNA translocation.

2.2 Introduction to mRNA and tRNA Translocation on the Ribosome
Protein biosynthesis requires many large-scale rearrangements in the
ribosome as each amino acid is added to a growing polypeptide chain.
Positioning of tRNA on the ribosome is proposed to occur through a ratcheting
mechanism. Central to this mechanism is a rotation of the small ribosomal
subunit relative to the large subunit (Fig. 2-1A) (Frank et al., 2007; Horan and
Noller, 2007) that occurs in all stages of translation—initiation, elongation,
termination, and ribosome recycling (Frank et al., 2007)—and is targeted by
clinically useful antibiotics (Ermolenko et al., 2007; Johansen et al., 2006). For
example, after each peptide bond is formed an ~8° intersubunit rotation results in tRNAs bound in the aminoacyl-tRNA and peptidyl-tRNA binding sites (A site and P site, respectively), moving into the P site and exit-tRNA site (E site) on the large ribosomal subunit (Fig. 2-1B). From this hybrid state of tRNA binding (Fig. 2-1B) (Frank et al., 2007; Moazed and Noller, 1989b), the tRNAs are then translocated to the P site and E site on the small subunit.

In addition to intersubunit rotation, ratcheting also involves a nearly orthogonal rotation of the head domain of the small ribosomal subunit (Fig. 2-1C) that plays a role in controlling the position of tRNAs within the ribosome (Allen and Frank, 2007; Schuwirth et al., 2005; Spahn et al., 2004). As with intersubunit rotation, movement of the head domain is a target for clinically useful antibiotics. Swiveling of the head domain relative to the body of the small subunit may also be required for the intrinsic helicase activity of the ribosome in unwinding secondary structure in mRNA (Borovinskaya et al., 2007; Takyar et al., 2005). Rotations of up to 14° allow the head domain to change its position by 20 Å or more at the ribosomal subunit interface, or the width of a tRNA substrate (Schuwirth et al., 2005).

The molecular basis for how the ribosomal subunits rotate with respect to each other while remaining stably associated remains unknown (Allen and Frank, 2007; Cornish et al., 2008). Furthermore, the precise timing of movements of the small subunit head domain during ratcheting are not clear because the head domain can move independently of the body and platform domains of the small subunit (Frank et al., 2007; Schuwirth et al., 2005; Spahn et al., 2004). In the following pages, three x-ray crystal structures of the Escherichia coli 70S ribosome are presented that reveal intermediates along the ratcheting pathway. These structures show that the ribosome can rearrange the interface between the ribosomal subunits in discrete steps and suggest how these rearrangements may direct tRNAs into hybrid states of binding that are essential for mRNA and tRNA translocation.

2.3 Results and Discussion

2.3.1 Structural Characterization of a New Intermediate Ratcheted Conformation of the Ribosome

Using new crystal forms, we determined x-ray crystal structures of the E. coli 70S ribosome in the absence of ligands, with mRNA and an anti-codon stem-loop mimic of tRNA^Met_f (ASL^Met_f) bound in the P site of the small (30S) subunit, or with mRNA and two ASL mimics of tRNA^Phe_f (ASL^Phe_f) bound in the A and P sites of the 30S subunit (3.5 Å, 4.0 Å, and 4.0 Å resolution, respectively). One of the two copies of the ribosome in each crystal structure resembles previously determined high-resolution structures of the ribosome (Berk et al., 2006; Schuwirth et al., 2005). In the other copies of the ribosome in each structure, the small subunit of the ribosome is rotated to an intermediate position with respect to the large subunit when compared with structures of the ribosome determined
previously. In the new conformation, the small subunit is rotated by 3° to 6° relative to its position in a post-initiation state of the ribosome, in which initiator tRNA is bound in the P site (defined here as state R0) (Berk et al., 2006; Selmer et al., 2006), and 2° to 3° relative to the ribosome with tRNAs bound in the P/P and E/E sites (here denoted state R1) (Selmer et al., 2006). In ribosomes in which tRNA occupies a hybrid P/E binding site (here called state RF) (Fig. 2-1A and 2-1B) (Frank et al. 2007; Connell et al. 2007; Agirrezabala et al. 2008; Julian et al. 2008), the small subunit is rotated by an additional 2° to 4° relative to the rotational state described here (Fig. 2-2E), which we term state R2. The ribosome can therefore adopt at least four stable states of intersubunit rotation, R0, R1, R2, and RF.

In state R2, the central contacts or “bridges” between the ribosomal subunits (Frank et al., 2007; Schuwirth et al., 2005) are nearly indistinguishable from those observed in ribosomes in states R0 and R1 (Fig. 2-3A) (Berk et al., 2006; Korostelev et al., 2008a; Laurberg et al., 2008; Schuwirth et al., 2005; Selmer et al., 2006; Weixlbaumer et al., 2008). These bridges include contacts between ribosomal RNA (rRNA) elements in the small and large subunits (16S rRNA and 23S rRNA, respectively) that are near the tRNA binding sites. The largest change in the central bridges occurs in bridge B2a, which is adjacent to the mRNA decoding site (Fig. 2-3A). In this region, nucleotide A1913 in 23S rRNA and nucleotides A1492 to A1493 in helix h44 of 16S rRNA adopt different conformations depending on the tRNA occupancy of the A site, as observed in other structures (Berk et al., 2006; Schuwirth et al., 2005; Selmer et al., 2006). To maintain contacts in bridges at the center of the interface (Fig. 2-3A) during subunit rotation, helix h44 in the small subunit bends near 16S rRNA helix h14 (Fig. 2-3B).

In contrast, key bridges between the platform of the 30S subunit and the 50S subunit (B4 and B7a) are shifted halfway to their position in the RF state (Figs. 2-2 and 2-3A) (Agirrezabala et al., 2008; Connell et al., 2007; Frank et al., 2007). In the structures observed here, the platform of the 30S subunit rotates about the helical axis of the top of helix h44 in 16S rRNA. The rotation exposes nucleotide A702 in 16S rRNA to solvent, whereas it is buried in the minor groove of H68 in 23S rRNA in high-resolution structures of states R0 and R1 (Fig. 2-3C) (Berk et al., 2006; Korostelev et al., 2008a; Laurberg et al., 2008; Selmer et al., 2006; Weixlbaumer et al., 2008). In 16S rRNA, nucleotide A702 is protected from chemical probes when tRNAs are bound in the A/A and P/P sites (Moazed and Noller, 1989b). However, when tRNAs occupy hybrid binding sites (A/P and P/E) (Fig. 2-1B), nucleotide A702 becomes exposed to chemical probes (Moazed and Noller, 1989b) and bridge B7a is rearranged (Agirrezabala et al., 2008; Connell et al., 2007; Frank et al., 2007). Exposure of A702 to solvent suggests that the conformation of the ribosome in state R2 is at least partway to the fully rotated state (RF) that accommodates tRNA binding in hybrid A/P and P/E sites (Fig. 2-1B). Apart from protein S15 in bridge B4, which is also shifted halfway to its position in state RF (Agirrezabala et al., 2008; Connell et al., 2007; Frank et al.,
2007), most of the remainder of the 30S platform does not make direct contact with the large subunit (Fig. 2-3A). Limited contacts probably allow large shifts in the position of the interface in this region.

Contacts between the 30S subunit head domain and the 50S subunit have been shown to adopt many different configurations. In states R₀ and R₁, protein L5 in the central protuberance of the 50S subunit contacts the N-terminal lobe of protein S13 in the 30S head domain when it is centered over the 30S P site (bridge B1b) (Berk et al., 2006; Frank et al., 2007; Selmer et al., 2006). In the R₁ state of the ribosome, the head domain of the 30S subunit is shifted so that protein S13 forms a key interaction between its long central α-helix and protein L5 in the large subunit (Cukras and Green, 2005; Frank et al., 2007; Hoang et al., 2004). In the structure of the ribosome in state R₂ with no bound ligands, the key interaction between proteins L5 and S13 (Cukras and Green, 2005; Hoang et al., 2004) is essentially indistinguishable from that in the fully rotated ribosome (Fig. 2-4B) (Agirrezabala et al., 2008; Connell et al., 2007; Frank et al., 2007). To enable the contact, the head of the small subunit is swiveled toward the E site by 11° relative to its conformation when the head domain is aligned with the 30S subunit P site (Figs. 2-1C and 2-4A) (Selmer et al., 2006), whereas the head domain is swiveled by only 5° in the R₁ state when tRNAs are bound in the hybrid A/P and P/E sites (Figs. 2-1B and 2-4B) (Agirrezabala et al., 2008; Connell et al., 2007; Frank et al., 2007). Cryogenic electron microscopy (cryo-EM) reconstructions of the yeast ribosome in the R₁ state reveal that tRNA bound in the P/E site remains associated with the head domain of the small subunit as the head domain adopts an “open” configuration required for tRNA transit to the small subunit E site (Frank et al., 2007). Using the head domain of the small subunit as a guide, we modeled full-length A/P and P/E tRNAs (Agirrezabala et al., 2008) in the ribosome in state R₂. Based on the model, tRNAs could bind in the hybrid A/P and P/E sites in the R₂ state because of swiveling of the head domain of the small subunit by ~11°, as observed in the apo-70S structure (Fig. 2-4C).

2.3.2 Interactions Between the Anticodon Stem Loop of tRNA and the 30S Subunit Control tRNA Positioning

Contacts between the ribosome and the ASL portion of tRNA may oppose movement of tRNAs into the hybrid state of binding. The ASL portion of P-site tRNA has been proposed to stabilize the 30S subunit head domain centered over the P site (0° rotation) and prevent frame-shifting (Berk et al., 2006). ASL binding to the ribosome in state R₂ also centers the head domain of the small subunit over the P site, as seen in state R₀ (Berk et al., 2006). This is true regardless of whether an ASL is bound in the A site or not. In state R₂, centering of the head domain over the P site would leave the acceptor end of P-site tRNA positioned partway between the P and E sites on the large subunit (Fig. 2-4D), which is presumably an unstable configuration except for translation initiation (Allen et al., 2005). To counterbalance the stabilizing effect of the ASL on the positioning of
the head domain, bridge B1b between proteins S13 and L5 and contacts between the large subunit and the elbow and acceptor ends of tRNA (Frank et al., 2007; Joseph and Noller, 1998; Lill et al., 1989; Munro et al., 2007; Pan et al., 2007) may favor 30S subunit head rotation and P-site tRNA movement into the hybrid P/E site in states R₂ and R₉, which is in agreement with the in vivo importance of the α-helical region of S13 involved in the bridge (Cukras and Green, 2005; Hoang et al., 2004) and the requirement of full-length P-site tRNA in order for translocation to occur (Joseph and Noller, 1998). Also consistent with our structural model of hybrid state formation, single-molecule fluorescence resonance energy transfer (FRET) experiments have shown that ribosomes lacking substrates or complexed with a P-site ASL^{Met}_{1} exhibit spontaneous subunit rotation, but not with the same rate or efficiency as when full-length P-site tRNA is bound (Cornish et al., 2008).

2.3.3 Structural and Single Molecule Data Suggest Translocation Proceeds Through Discrete Intermediates

The structures of the E. coli 70S ribosome presented here reveal that in addition to the known R₀, R₁, and R₉ states, the ribosome can adopt an intermediate state of subunit rotation, state R₂. This state would likely occur transiently, before the ribosome adopts a fully rotated conformation (Agirrezabala et al., 2008; Cornish et al., 2008; Frank et al., 2007). Based on the structures presented above, we propose that during ratcheting, which combines inter-subunit rotation and rotation of the small subunit head domain, key bridges between the ribosomal subunits rearrange in a step-wise manner. Ratcheting likely begins with the 30S subunit body, continuing with the 30S platform and head domains, and completes with rearrangement of the central bridges (Fig. 2-4E). Such a stepwise rearrangement would assist the ribosome in making large shifts at the interface without fully destabilizing the subunit interface. In addition, the multiple conformations of the head domain of the 30S subunit would help to position tRNAs on the ribosome during ratcheting.

Intriguingly, single-molecule FRET studies have shown that P-site tRNA fluctuates between the P/P and P/E sites at a faster rate than intersubunit rotation (Cornish et al., 2008; Munro et al., 2007). The ability of tRNAs to adopt a hybrid state of binding in state R₂ may, in part, explain the different rates between these processes. Fluctuation of tRNAs between the P/P and P/E states would not require the full extent of intersubunit rotation but could occur in state R₂ because of swiveling of the 30S subunit head domain and the inherent flexibility of P-site tRNA (Korostelev et al., 2006; Selmer et al., 2006). High-resolution structures of the ribosome in different rotated states with intact tRNAs will be required to complete our molecular understanding of the large-scale conformational rearrangements in the ribosome that allow ratcheting and protein synthesis to occur. To that end, a recent EM experiment imaging the ribosome during a reverse translocation reaction revealed several new intermediate conformations of the ribosome, reminiscent of the R₂ state proposed here (Fischer et al. 2010).
2.4 Methods

2.4.1 Ribosome Purification and Crystallization
Ribosomes were purified from *E. coli* strain MRE600 using sucrose gradient centrifugation, similar to the method described by Blaha et al. (Blaha et al., 2000) with one distinct difference. *E. coli* ribosomes contain a small ribosomal subunit protein, termed S1, which has a molecular weight of 68 kDa and contains 6 repeats of a five stranded beta-barrel motif, sequence analysis reveals. It has been shown to have an extended structure in solution (Laughrea and Moore, 1977), as would be expected for such a domain structure. Due to the extended nature of the protein and its location on the 30S subunit (Sengupta et al., 2001) it inhibits tight packing of 70S ribosomes adjacent to each other, and thus inhibits crystallization. During purification S1 is depleted from ribosomes by incubation of a polypyrimidine (poly-U) nucleic acid, followed by separation of the polypyrimidine and S1 containing fraction, from ribosomes by ultracentrifugation.

Ribosome crystallization is performed at 18°C using microbatch 96-well plates and buffers containing 4-5% 2-methyl-2,4-pentanediol (MPD), 3.9-4.4% PEG 8000, 3.8 mM MgCl$_2$, 380 mM NH$_4$Cl, 5.5 mM putrescine, 5 mM spermidine, 10 mM Tris plus 20 mM MES, pH=6.5-7.0, and 0.25 mM EDTA. For complexes containing mRNA and ASL$^{\text{Metf}}$, 5 µM mRNA of sequence 5′-AUGUUU-3′ and 10 µM of ASL$^{\text{Metf}}$ (nucleotides 27-43 of tRNA$^{\text{Metf}}$) (Dharmacon) were included in the crystallization trials. For complexes containing mRNA and ASL$^{\text{Phe}}$, 5 µM mRNA of sequence U6 and 10 µM of ASL$^{\text{Phe}}$ (nts 27-43 of tRNA$^{\text{Phe}}$) were included. Crystals typically appear in 24 hours and grow to maximum size (~100 x ~100 x ~800 µm) in 2 to 4 days. The crystals are then stabilized with crystallization buffer containing 7% MPD, 7% PEG 8000 and 24% PEG 400, pH=4.8, to allow cryocooling of the crystals in liquid nitrogen.

2.4.2 Diffraction Data Collection
X-ray diffraction data were collected at the NE-CAT beamline 24-ID-E of the Advanced Photon Source (apo ratcheted structure) and at the SIBYLS beamline 12.3.1 of the Advanced Light Source (ASL containing structures). In both cases crystals were placed in a 100K nitrogen stream, exposed to x-rays of 1.11 Å or 0.97 Å wavelength using a 0.1-0.3 degree oscillation per image. Diffraction was measured on a ADSC q315 ccd detector and integrated with the Denzo/Scalepack software suite. Table 2-1 shows the statistics for the reflections for each of the three datasets.

2.4.3 Refinement and Model Building
The two copies of the 70S ribosome in the crystallographic asymmetric unit were located using the program Phaser (McCoy et al., 2005) and atomic-resolution
structures of the *E. coli* ribosome (Schuwirth et al., 2005). The resulting structural models were then refined using rounds of manual rebuilding (Jones et al., 1991) and torsional dynamics (Brünger et al., 1998). Additional stereochemical restraints were used to maintain stable rounds of refinement during early cycles of modeling, as described (Schuwirth et al., 2005). The final positional and TLS refinement of the structures was carried out using Phenix (Adams et al., 2002). Electron density maps were generated using the programs Pirate (Cowtan, 2000) and CNS (Brünger et al., 1998).

### 2.4.4 Superpositions and Rotation Calculations

Comparisons to atomic-resolution structures of the ribosome, and to structural models of the intact ribosome refined against cryo-EM density maps, were carried out by least-squares superposition in the program O (Jones et al., 1991), using ribose C1’ positions in nucleotides. Superpositions to identify the relative position of the small and large subunits in the ribosome used the large subunit as the frame of reference (Frank et al., 2007). Superpositions of the small subunits with each other utilized conserved 16S rRNA nucleotides in the body, platform and head domains of the small subunit (2). Comparisons to cryo-EM reconstructions of the *E. coli* or *T. thermophilus* 70S ribosome were made after first refining the 3.4 Å structures presented here, plus tRNAs and mRNA taken from the *T. thermophilus* 70S ribosome (Connell et al., 2007; Selmer et al., 2006), as a series of rigid bodies against structure factors (amplitudes and phases) derived from a series of cryoEM reconstructions (Agirrezabala et al., 2008; Allen et al., 2005; Connell et al., 2007; Gabashvili et al., 2000; Villa et al., 2009). The rastering of the cryo-EM reconstruction in (Agirrezabala et al., 2008) was reduced ~3.2%, and that in (Gabashvili et al., 2000) by ~1.9% to minimize the refinement residual.

Discussions of representative ratcheted states rely on structures of post-initiation complexes (Berk et al., 2006; Gabashvili et al., 2000), and post-termination complexes (Korostelev et al., 2008a; Laurberg et al., 2008; Weixlbaumer et al., 2008) for state R0; mRNA decoding and pre- or post-translocation states (Agirrezabala et al., 2008; Selmer et al., 2006; Villa et al., 2009) for state R1; and ribosomes with tRNAs in hybrid A/P and/or P/E states (Agirrezabala et al., 2008; Connell et al., 2007) for state RF. The distinction between states R0 and R1 has not been noted before, and is most pronounced with respect to the positioning of the body of the 30S subunit (Table S3). Rotation angles presented in Table S3 correspond to roughly 0.5 Å per degree for bridges B2a and 1.2 Å per degree at bridge B7a. The rotation angles for the body domain do not include opening and closing of the domain around the 30S A site, a separate and nearly orthogonal degree of freedom described in (Ogle et al., 2002) in the context of mRNA decoding. Angles and distances given for the rotation of the head domain were calculated from 30S subunit structures taken from 70S ribosomes in different states (Agirrezabala et al., 2008; Connell et al., 2007; Gabashvili et al., 2000; Laurberg et al., 2008; Selmer et al., 2006) and
superimposed by means of their platform domains. A rotation of 0° is defined as centering the head domain over the 30S P site.

3. Structures of the Escherichia coli Ribosome with Antibiotics Bound Near the Peptidyl Transferase Center Explain Spectra of Drug Action


3.1 Abstract

Differences between the structures of bacterial, archaeal, and eukaryotic ribosomes account for the selective action of antibiotics. Even minor variations in the structure of ribosomes of different bacterial species may lead to idiosyncratic, species-specific interactions of the drugs with their targets. Although crystallographic structures of antibiotics bound to the peptidyl transferase center or the exit tunnel of archaeal (*Haloarcula marismortui*) and bacterial (*Deinococcus radiodurans*) large ribosomal subunits have been reported, it remains unclear whether the interactions of antibiotics with these ribosomes accurately reflect those with the ribosomes of pathogenic bacteria. Here we report X-ray crystal structures of the *Escherichia coli* ribosome in complexes with clinically important antibiotics of four major classes, including the macrolide erythromycin, the ketolide telithromycin, the lincosamide clindamycin, and a phenicol, chloramphenicol. Binding modes of three of these antibiotics show important variations compared to the previously determined structures. Biochemical and structural evidence also indicates that interactions of telithromycin with the *E. coli* ribosome more closely resemble drug binding to ribosomes of bacterial pathogens. The present data further argue that the identity of nucleotides 752, 2609, and 2055 of 23S ribosomal RNA explain in part the spectrum and selectivity of antibiotic action.

3.2 Introduction to Mechanistic Studies of Protein Synthesis Inhibition

Antibiotics are small organic molecules synthesized by fungi and bacteria that can inhibit the growth of other microorganisms (Cundcliffe, 2000). The ribosome is a major target of antibiotics, which affect nearly all steps of protein synthesis (Sohmen et al., 2009). The peptidyl transferase center (PTC) of the ribosome is inhibited by a chemically diverse group of compounds including lincosamides and phenicols (Figs. 3-1A, 3-1B, 3-1C) (Polacek and Mankin, 2005). Despite the chemical dissimilarities of these compounds they share overlapping mechanisms of inhibition by preventing proper orientation of tRNA in the PTC and interfering with peptide bond formation. Another important group of drugs, macrolides and their modern ketolide derivatives, bind in the exit tunnel of the large ribosomal subunit and inhibit extrusion of the nascent peptide, leading to peptidyl tRNA drop-off (Figs. 3-1B, 3-1C) (Mankin, 2008; Tu et al., 2005; Vester and Douthwaite, 2001).
Studies aimed at mechanistically understanding how antibiotics inhibit protein synthesis have an approximately forty year history. Early studies used radio-labeled antibiotics incubated with ribosomal subunits to determine which subunit, 30S or 50S, the antibiotic bound to, the stoichiometry of binding and the affinity. Experiments using chemical footprinting of rRNA allowed specific regions in the secondary structure of rRNA to be assigned functional roles such as A-site tRNA binding or P-site tRNA binding (Moazed and Noller, 1989a). Meanwhile, footprints for antibiotics could be obtained and hypotheses made about mechanisms of inhibition if antibiotic footprints overlapped footprints for a functional site (Moazed and Noller, 1987, 1989a). Footprinting revealed, for example, that chloramphenicol protects 23S rRNA residue A2451, which is also protected by aminoacyl tRNA, but not de-acylated tRNA, indicating that the antibiotic inhibits peptide bond formation by preventing the proper positioning of the attacking amino acid and the nascent peptide (Moazed and Noller, 1989a). Footprinting, however, could not provide information about which chemical components of an antibiotic interacted with which residues of rRNA or generate three dimensional models.

NMR experiments using fragments of rRNA from the 16S A-site, which assays showed retained a functional structure, provided early three dimensional understanding of how antibiotics bound rRNA (Fourmy et al., 1996). X-ray crystal structures of the ribosomal subunits at atomic resolution followed soon after, opening up the flood gates for structural investigation of antibiotic rRNA interactions. The first publications reporting antibiotics bound to the 30S subunit appeared in 2000 and 2001 (Broderson et al. 2000; Pioletti et al., 2001) while 50S subunit antibiotic complexes appeared in 2002 (Hansen et al., 2002). These experiments made great strides toward explaining the mechanism of inhibition for a number of ribosome targeting antibiotics, rationalized resistance mutations and brought the possibility of rationally designed antibiotics within reach. Yet despite the power of these x-ray crystal structures as new scientific tools, for some compounds, such as those which bind in the peptide exit tunnel or PTC uncertainty concerning their interactions with rRNA persists. This is due to many significant disagreements between the reports for antibiotics bound to either the D. radiodurans or H. marismortui 50S ribosomal subunits (Wilson et al., 2005). These differences include the conformation of the macroline ring, the conformation of the alkyl aryl arm of telithromycin, the orientation of the pyrroldinyl moiety of clindamycin and the two non-overlapping binding sites observed for chloramphenicol (Beresio et al., 2003; Hansen et al., 2002; Hansen et al., 2003; Schluenzen et al., 2001; Tu et al., 2005). There are a number of possible ways to explain these discrepancies, but one in particular, is the differing rRNA sequences in these organisms (Fig. 3-1C). H. marismortui nor D. radiodurans contain differences with respect to each other and with respect to medically relevant pathogenic bacteria.

To address the differences which persist between the H. marismortui and D. radiodurans structural data, I solved structures of four antibiotics bound to the
E. coli ribosome: erythromycin, telithromycin, clindamycin and chloramphenicol. Since the E. coli ribosome has rRNA sequences similar to bacterial species of medical interest, these data give a more accurate picture of the interactions between antibiotics and the large ribosomal subunit of pathogenic bacteria. Together with biochemical data probing the interactions of antibiotics with the PTC and exit tunnel, the present structures reveal how rRNA sequence differences contribute to the spectrum of activity for antibiotics and offer new clues as to why these compounds do not inhibit cytoplasmic eukaryotic ribosomes.

3.3 Results and Discussion

3.3.1 The Structure of Erythromycin Bound to the E. coli Ribosome is Similar to the Structure of the Compound Bound to the H. marismortui Ribosomal Subunit

The original macrolide antibiotic in clinical use since the 1950s, erythromycin is composed of a 14-membered macrolactone ring, with carbohydrates at positions 3 and 5 (Fig. 3-1A). The desosamine sugar at position 5, which contains a dimethyl amine that is crucial for binding to the ribosome, makes contact with A2058 in 23S rRNA, the most commonly mutated nucleotide in resistant bacteria. The 14-atom macrolactone ring of erythromycin serves as the scaffold for several semisynthetic compounds in clinical use, with various appendages attached to the ring. The macrolactone ring was initially reported in two different conformations, “folded-in” and “folded-out” when bound to D. radiodurans and H. marismortui 50S subunits, respectively (Schluenzen et al., 2001). However, the existence of the folded-in conformation for erythromycin when bound to the ribosome has been questioned because a putatively lower energy folded-out conformation of erythromycin exists in the crystal structure of the free compound (Tu et al., 2005). In the structure of erythromycin bound to the E. coli 70S ribosome, we observed difference electron density for the drug in excellent agreement with its position bound to the H. marismortui 50S ribosomal subunit containing a G2058A mutation (Tu et al., 2005) and with its conformation in the crystal structure of the free compound (Fig. 3-2A) (Stephenson et al., 1997). A slight movement of the antibiotic relative to its position when bound to the G2058A mutant H. marismortui 50S subunit is visible, possibly due to a movement of rRNA helix H73 and the adjacent nucleotides in the E. coli ribosome, relatively to their position in the H. marismortui 50S subunit (Fig. 3-2B). In spite of this spatial translocation, the drug maintains its contacts with A2058, which involves a hydrogen bond between the desosamine hydroxyl and the N1 atom of A2058, and tight packing of the hydrophobic face of the lactone ring against nucleotides 2611 and 2057 in the peptide exit tunnel wall. Notably, H. marismortui contains a G-C base pair C2057-G2611 in the opposite polarity of the E. coli base pair G2057-C2611, but this seems not to affect the interaction with erythromycin significantly (Fig. 3-1C and Fig. 3-2B).
3.3.2 The Structure of Telithromycin Bound to the E. coli Ribosome Reveals a New Interaction with rRNA Explaining Resistance Mutations

Telithromycin belongs to the family of ketolide antibiotics that represent the newest generation of macrolides. In telithromycin, a carbonyl group replaces the C3 cladinose sugar (Fig. 3-1A), which in macrolides is necessary for ribosome stalling and regulating the induction of resistance genes (Vazquez-Laslop et al., 2008). Similar to other clinically relevant ketolides, telithromycin contains an alkyl-aryl arm attached to a carbamate heterocycle that involves the C11 and C12 positions of the ketolide macrocycle. This moiety increases the affinity of the ketolide scaffold for the ribosome by several hundred-fold, demonstrating that it is an important pharmacophore (Hansen et al., 1999). The alkyl-aryl arm of telithromycin was seen in two distinct conformations in prior ribosome crystal structures. It was folded back over the macrolactone ring when bound to the G2058A H. marismortui 50S subunit, or interacting with rRNA further down the peptide exit tunnel when bound to the D. radiodurans 50S subunit (Fig. 3-3A) (Berisio et al., 2003; Tu et al., 2005). Importantly, neither structure can easily explain telithromycin resistance mutants at residue U2609, telithromycin protection of A752 in RNA footprinting experiments, or the fact that deletion of A752 or mutations in its vicinity lead to resistance (Canu et al., 2002; Garza-Ramos et al., 2001; Novotny et al., 2004; Xiong et al., 2005; Xiong et al., 1999).

In the E. coli ribosome, in contrast to H. marismortui or D. radiodurans, nucleotides A752 and U2609 form a base pair that bridges domains II and V in 23S rRNA. Notably, in the structure of telithromycin bound to the E. coli ribosome, the alkyl aryl arm stacks on the A752-U2609 base pair, a conformation not observed in prior structures (Figure 3-3B, Fig. 3-3C). By contrast, the position of the macro lactone ring remains in essentially the same conformation observed when telithromycin is bound to the G2058A H. marismortui 50S subunit (Fig. 3-3A) (Berisio et al., 2003; Tu et al., 2005). The A752-U2609 base pair, which exists in E. coli and many other eubacteria but not in H. marismortui or D. radiodurans (Fig. 3-3C), provides a surface for the entire face of the alkyl aryl arm to engage in a stacking interaction that likely favors drug binding. Stacking of the alkyl-aryl arm of telithromycin on the A752-U2609 base pair would also likely lead to protection of A752 from chemical probes by stabilizing the base pair interaction. To test this model, we probed E. coli ribosomes in solution by RNA footprinting for telithromycin protections of this base pair. We also examined H. halobium (a close relative of H. marismortui), D. radiodurans and S. aureus ribosomes. Binding of telithromycin to E. coli or S. aureus ribosomes protected A752 from chemical modification (Fig. 3-4). By contrast, binding of telithromycin to D. radiodurans and H. halobium ribosomes did not protect the corresponding nucleotide, although both compounds were bound in all cases (Fig. 3-4). Given the sequence conservation of the A752-U2609 base pair among many eubacteria, the structural and biochemical data obtained here suggest that the interactions of telithromycin with the E. coli ribosome likely reflect those that
occur when telithromycin binds the ribosomes of medically relevant eubacterial species. Furthermore, the interaction between the alkyl aryl arm and the A752-U2609 base pair helps to explain why deletion of A752 or mutations of U2609 provide resistance to telithromycin (Canu et al., 2002; Garza-Ramos et al., 2001; Novotny et al., 2004).

3.3.3 The Structure of Clindamycin Bound to the E. coli Ribosome Reveals that the Compound Inhibits A-site tRNA Positioning

Clindamycin is a semi-synthetic derivative of the lincosamide class of compounds used clinically to treat gram-positive bacterial infections. Structural models of clindamycin bound to both the G2058A H. marismortui and D. radiodurans 50S ribosomal subunits (Schluenzen et al., 2001; Tu et al., 2005) agreed roughly in the placement of the galactose sugar of clindamycin but differed in the positioning of the propyl pyrrolidinyl moiety of the antibiotic, the portion that is hypothesized to interfere with A-site tRNA positioning (Tu et al., 2005). In the models of clindamycin bound to the H. marismortui and D. radiodurans 50S subunits, the pyrrolidinyl propyl group is rotated by 180° in one structure relative to the other (Fig. 3-4A). Structural studies of the ribosome containing aminoacylated A-site and P-site tRNAs, or oligonucleotide mimics of the tRNAs, show that the pyrrolidinyl propyl group, as modeled in the H. marismortui complex, would interfere with the positioning of A-site aminoacyl-tRNA (Voorhees et al., 2009). Unbiased difference electron density maps for clindamycin bound to the E. coli ribosome show density for the propyl group consistent with its position in the H. marismortui structure (Figs. 3-5A, 3-5B). Thus, clindamycin and other lincosamides likely interfere with A-site aminoacyl-tRNA binding, as proposed (Tu et al., 2005).

The location of the galactose ring, which forms numerous hydrogen bonds with A2058, A2059, G2505 and A2503 in both the G2058A H. marismortui and D. radiodurans 50S subunit structures, overlaps with the binding site of the desosamine sugar of macrolides and ketolides, explaining why lincosamides share resistance mutations with these antibiotic families. In the structure of the E. coli ribosome with clindamycin bound, difference electron density for the galactose ring agrees with the prior structural models for placement of the sugar (Fig. 3-5A). The interactions of clindamycin with 23S rRNA nucleotides A2058 and A2503 in E. coli are identical to those observed with the corresponding H. marismortui nucleotides. However, nucleotides 2504-2507 are shifted relative to their positions in the G2058A H. marismortui 50S subunit, leading to alternative interactions with clindamycin (Fig. 3-5D). The shift probably is due to the presence of A2055 in H. marismortui versus C2055 in E. coli, which stacks against U2504 in the structure. This stacking interaction, together with differences in the base pair partners of G2505 and U2609, alters the conformations that nucleotides in this region adopt in E. coli ribosomes. In the unbiased difference electron density map, a strong positive peak indicates a shift of G2505 and U2506 toward the drug molecule, with U2506 packing
perpendicular to the propyl group of clindamycin. Additional packing with C2452 leads to desolvation of the hydrophobic propyl group (Fig. 3-5C). The altered position of these nucleotides in *E. coli* ribosomes relative to their position in *H. marismortui* 50S subunits allows the bridging amine of clindamycin to hydrogen bond to the ribose O4’ of U2505, while preserving contacts with A2058 and A2503 (Figs. 3-5B, 3-5D). Thus, a comparison of the structures of clindamycin bound to the G2058A *H. marismortui* and *E. coli* ribosomes suggests that clindamycin forms fewer hydrogen bonds to rRNA in the *E. coli* structure, but maintains significant van der Waals contacts with the ribosome.

### 3.3.4 The Structure of Chloramphenicol Bound to the *E. coli* Ribosome Reveals Its Binding Site on the Eubacterial Ribosome

Prior structural studies identified one binding site for chloramphenicol in the *D. radiodurans* 50S subunit and a second distinct site in the *H. marismortui* 50S subunit (Hansen et al., 2003; Schluenzen et al., 2001). The differing positions of chloramphenicol are probably due to the fact that wild type *H. marismortui* is resistant to chloramphenicol, possibly due to the low affinity of the drug for its primary binding site (Mankin and Garrett, 1991). This intrinsic tolerance of archaea to chloramphenicol is likely due to rRNA sequence differences between archaea and bacteria, i.e. in nucleotides C2055 of H73, and U2609 and C2610 adjacent to the base of rRNA helix H73 (Fig. 3-1C). In the structure of chloramphenicol bound to the *E. coli* ribosome, we did not observe any difference electron density in the chloramphenicol binding site identified in the *H. marismortui* 50S subunit (Hansen et al., 2003). By contrast, clear positive difference electron density is located at the site of chloramphenicol binding reported in *D. radiodurans* 50S subunits, but this density is not entirely consistent with the reported orientation of chloramphenicol (Figs. 3-1B, 3-6B) (Schluenzen et al., 2001). In the *E. coli* ribosome complex, the nitrobenzene moiety of chloramphenicol overlaps with the position that would be occupied by the pyrrolidinyl propyl group of clindamycin (Figs. 3-1B, 3-5C, 3-6C). When compared to the structure of clindamycin bound to the *E. coli* ribosome, the base of U2506 is rotated such that the hydrophilic nitro group in chloramphenicol points into solvent. The nitrobenzene ring of chloramphenicol is stacked on C2452 (Fig. 3-6C) and positions one chlorine atom of chloramphenicol in a similar position to the chlorine in clindamycin (Fig. 3-5B). The other chlorine atom in chloramphenicol is in a position to contact the exocyclic amine of A2062, a residue that when mutated results in chloramphenicol resistance (Mankin and Garrett, 1991). Finally, the amine of chloramphenicol hydrogen bonds to the non-bridging phosphate oxygen of G2505 (Fig. 3-6C).

Since rRNA sequences in the region of chloramphenicol binding are conserved in both *E. coli* and *D. radiodurans*, sequence divergence cannot explain the different orientations of the antibiotic. Unless the second shell rRNA residues dramatically affect chloramphenicol binding, there is likely one principal mode of interaction between chloramphenicol and eubacterial ribosomes.
The unbiased difference electron density in the *E. coli* structure determination provides a much clearer view of details of chloramphenicol orientation than is provided by the electron density used to model chloramphenicol bound to the *D. radiodurans* 50S subunit (compare Fig. 3-5A with Fig. 1C of (Schluenz et al., 2001)). Furthermore, the structural model of chloramphenicol bound to the *E. coli* ribosome is at higher resolution and with a lower R<sub>free</sub> value (25%) when compared to the *D. radiodurans* model (R<sub>free</sub> = 32%) (Table 3-2). Finally, the interactions between chloramphenicol and rRNA in the orientation in the present model are more chemically favorable due to additional stacking interactions that should facilitate drug binding. Notably, in the present structural model of chloramphenicol bound to the *E. coli* 70S ribosome, the nitrobenzene ring stacks on C2452 in the same orientation that the methoxyphenyl ring of the related compound anisomycin stacks on C2452 when bound to the *H. marismortui* 50S subunit (Blaha et al., 2008).

### 3.3.5 Sequence divergence adjacent to the PTC determines the spectrum of efficacy for peptidyl transfer and peptide elongation inhibitors

The rRNA of the PTC is highly conserved throughout the 3 domains of life, explaining why many antibiotics which bind in or near the PTC can inhibit a wide range of species. However, many antibiotics which bind in this region are selective. For example erythromycin does not bind to eukaryotic or archaeal ribosomes except at extremely high concentrations. The identity of nucleotide 2058, adenosine in eubacteria, guanosine in eukaryotes and archaea, is thought to be a major contributor to this phenomenon (Tu et al., 2005). Conversely, wild type eubacteria are resistant to anisomycin while archaea and eukaryotes are sensitive (Hummel and Böck, 1987).

Our data reveal several rRNA residues that can account for the selectivity of antibiotic action. In the *E. coli* ribosome, 23S rRNA residues A752 and U2609 form a base pair that interacts with a key element of telithromycin, its extended 11,12 alkyl-aryl arm (Figs. 3-3A, 3-3B). Our data suggest that disruption of this base pair would decrease the affinity of telithromycin for the ribosome, consistent with mutations U2609C or deletion of A752 that lead to low level telithromycin resistance (Garza-Ramos et al., 2001; Novotny et al., 2004). In *D. radiodurans* and *H. marismortui*, this base pair does not exist, leading to a different conformation of the alkyl-aryl side chain (Berisio et al., 2003; Tu et al., 2005), and likely decreasing telithromycin affinity for the ribosome (Fig. 4).

Nucleotide C2055 in eubacteria is an adenosine in archaea and eukaryotes (Table 3-1) (2), and also seems to serve as an important determinant for the spectrum of action of A-site inhibitors such as chloramphenicol and clindamycin, as well as other antibiotics (Davidovich et al., 2007; Gurel et al., 2009). Our structures reveal that alteration of C2055, which stacks on U2504, to A, leads to a displacement of U2504 along with G2505, U2506 and C2507 (Figs. 3-1C, 3-4B). Thus the sequence difference at C2055 results in a change in the position of four universally conserved PTC nucleotides (Table 3-1).
structures of the *E. coli* ribosome show that chloramphenicol and clindamycin both interact with these nucleotides. G2505 hydrogen bonds to clindamycin while U2506 desolvates the propyl group of clindamycin (Figs. 3-4C, 3-4D). G2505 forms hydrogen bonds to chloramphenicol, interactions that are not possible in *H. marismortui*, given the conformation of this residue in *H. marismortui* ribosomal subunits (Fig. 3-4C). These structural data explain why archaeal and eukaryotic ribosomes bind lincosamides and chloramphenicol poorly. In fact, difference electron density for chloramphenicol is not observed in the eubacterial binding site even when *H. marismortui* ribosomal subunits are soaked in millimolar concentrations of the compound (Hansen et al., 2003). In addition, the mutation C2055A in eubacterial ribosomes increases the minimal inhibitory concentration (MIC) of chloramphenicol and clindamycin (Long et al., 2009). Furthermore, the C2055A mutation in *M. smegmatis* ribosomes leads to resistance to pleuromutilin antibiotics, which also bind the eubacterial PTC (Schlünzen et al., 2004).

The proposed role of 23S rRNA nucleotide 2055 as an antibiotic resistance spectrum determinant through its interaction with A-site cleft nucleotides 2504 and 2506-2507 suggest that mutations of these residues should also appear in mutants resistant to chloramphenicol and lincosamides. Despite the sensitive location of these nucleotides in the PTC and their universal conservation, mutations of nucleotide 2504 confer resistance to chloramphenicol (Blanc et al., 1981; Long et al., 2009) and increase the MIC of clindamycin (Long et al., 2009). The lack of posttranscriptional modification (pseudouridinylation) at this position renders bacteria hypersensitive to several peptidyl transferase inhibitors (Toh and Mankin, 2008).

Mutation of G2058A in archaeal ribosomes leads to a ~10⁴-fold improvement in macrolide binding to *H. marismortui* or *H. halobium* 50S subunits, suggesting that the identity of a single nucleotide could explain the insensitivity of archaeal and eukaryotic ribosomes towards macrolides (Bottger et al., 2001; Tu et al., 2005). However, in *S. cerevisiae* ribosomes carrying the G2058A mutation in their ribosomal RNA were still not inhibited by erythromycin, suggesting additional phylogenetic differences contribute to resistance (Bommakanti et al., 2008). Taken together with phylogenetic data, the present structural and biochemical data suggest that, in addition to position 2058, divergence of rRNA at positions 752, 2609, 2610 and 2055 also contributes to the species specificity of PTC and peptide elongation inhibitors. The availability of high-resolution structures of the ribosome and ribosomal subunits from archaea and divergent bacteria will now make it possible to probe these and other sequence determinants of PTC and peptide elongation inhibitors.

### 3.4 Materials and Methods

#### 3.4.1 Introduction of the Antibiotics into the Crystals
Ribosomes were purified as previously described previously (Schuwirth et al., 2005). Crystals were grown as previously described (Zhang et al., 2009).
antibiotic soaking experiments, the cryoprotection buffer was supplemented with 48 μM of telithromycin or 100 μM of either erythromycin, chloramphenicol or clindamycin. Erythromycin, chloramphenicol and clindamycin were obtained from Sigma. Telithromycin was a gift from Cempra Pharmaceuticals. The telithromycin stock solution in 50% acetic acid was diluted ~800-fold in cryoprotection buffer for soaks. Erythromycin and chloramphenicol stock solutions were made in ethanol, and diluted 100-fold to working concentrations in cryoprotection buffer. Clindamycin was dissolved in DMSO and diluted 100x in cryoprotection buffer for soaking experiments. In all four cases incubation of the crystals with antibiotic took place overnight. Crystals were then flash frozen in liquid nitrogen.

3.4.2 Diffraction Data Collection, Refinement and Model Building
X-ray diffraction data were measured at beamlines 8.3.1 and 12.3.1 of the Advanced Light Source, Lawrence Berkeley National Laboratory, using 0.1-0.3 degree oscillations at 100K and recorded on an ADSC Q315 detector. X-ray reflections were reduced and scaled using HKL2000. Difference electron density (F_{obs}–F_{calc}) maps were calculated using the phenix.refine module of the PHENIX software suite (Adams et al., 2010). Antibiotics were positioned based on the unbiased difference electron density obtained in each experiment. If necessary, changes in the coordinates for rRNA nucleotides dictated by the difference electron density were made using Coot (Emsley and Cowtan, 2004) and phenix.refine was used for positional and atomic displacement parameter (ADP) refinement of the model. The crystals contain two copies of the ribosome in the asymmetric unit, with molecule 1 (PDB coordinates 3OAT, 3OFR, 3OFC, 3OFZ for the 50S subunit in complex with telithromycin, erythromycin, chloramphenicol, and clindamycin, respectively) exhibiting lower ADP values and clearer electron density than molecule 2. While difference electron density for antibiotics was observed in both molecule 1 and molecule 2 of the crystal in each experiment, coordinates for antibiotics were only modeled into molecule 1.

3.4.3 rRNA Sequence Alignments
Ribosomal RNA sequence alignments were made using MUSCLE (Edgar, 2004) or obtained from the Comparative RNA Website (2). Coordinate superpositions were performed in PyMOL using the C1' atoms of domain V of 23S rRNA, excluding regions where insertions or deletions occur.

3.4.4 Chemical Probing Experiments
Ribosomes and large ribosomal subunits were isolated from E. coli (strain JM109) or Staphylococcus aureus (strain RN4220) using standard protocols described in (Moazed and Noller, 1989a) and (Merryman, 1998). Large ribosomal subunits prepared from Deinococcus radiodurans were a gift of Dr. Franchescchi and Dr. Skripkin (Rib-X, Inc). In order to increase affinity of macrolides for the archaeal large ribosomal subunits, G2058 in the 23S rRNA gene (E. coli
numbering) of the single ribosomal operon of *Halobacterium halobium* was mutated to A. This mutation was introduced together with the selective anisomycin resistance mutation C2452U using the procedure described in (Mankin et al., 1992). Haloarchaeal ribosomes were isolated as previously described (Tan et al., 1996). Chemical probing of interactions of erythromycin and telithromycin with bacterial and haloarchaeal large ribosomal subunits was carried out using procedures described in (Mankin et al., 1992) and in (Tan et al., 1996) with ribosomes and drugs present at 200 nM and 50 µM, respectively. Ribosomal subunits, vacant or complexed with antibiotics, were modified with dimethylsulfate and the distribution of modified residues was analyzed by primer extension.

4. Structure of the *E. coli* Ribosome Bound to the New Ketolide CEM-101


4.1 Abstract

Following is a characterization of the mechanism of action and the drug-binding site of a novel ketolide, CEM-101, which belongs to the latest generation of macrolide antibiotics. CEM-101 shows high affinity for the ribosomes of Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria. The ketolide shows high selectivity in its inhibitory action and readily interferes with synthesis of a reporter protein in the bacterial but not eukaryotic cell-free translation system. Binding of CEM-101 to its ribosomal target site was characterized biochemically and by X-ray crystallography. The X-ray structure of CEM-101 in complex with the *E. coli* ribosome shows that the drug binds in the major macrolide site in the upper part of the ribosomal exit tunnel. The lactone ring of the drug forms hydrophobic interactions with the walls of the tunnel, the desosamine sugar projects toward the peptidyl transferase center and interacts with the A2058/A2509 cleft, and the extended alkyl-aryl arm of the drug is oriented down the tunnel and makes contact with a base pair formed by A752 and U2609 of the 23S rRNA. The position of the CEM-101 alkyl-aryl extended arm differs from that reported for the side chain of the ketolide telithromycin complexed with either bacterial (*Deinococcus radiodurans*) or archaeal (*Haloarcula marismortui*) large ribosomal subunits but closely matches the position of the side chain of telithromycin complexed to the *E. coli* ribosome. Difference in the chemical structure of the side chain of CEM-101 in comparison with the side chain of telithromycin and the presence of the fluorine atom at position 2 of the lactone ring likely account for superior activity of CEM-101. The results of chemical probing suggest that the orientation of the CEM-101 extended side chain observed in the *E. coli* ribosome closely resembles its placement in *Staphylococcus aureus* ribosomes and thus likely accurately reflects interaction
of CEM-101 with the ribosomes of the pathogenic bacterial targets of the drug. Chemical probing further demonstrated weak binding of CEM-101, but not of erythromycin, to the ribosome dimethylated at A2058 by the action of Erm methyltransferase.

4. Introduction to Ketolide Antibiotics

Macrolide antibiotics bind to the large ribosomal subunit and inhibit protein synthesis by blocking the path of the nascent peptide in the exit tunnel (Gaynor and Mankin, 2003; Gentry and Holmes, 2008). The chemical structure of the prototype macrolide erythromycin A is represented by a 14-atom lactone ring decorated with C3-cladinose and C5-desosamine sugar residues (Figure 4-1). Macrolides of the subsequent generations differed in the structures of the lactone as well as the number, composition, and sites of attachment of the side chains (Franceschi et al., 2004; Sutcliffe, 2005).

The binding site of macrolides in the ribosome is composed of 23S rRNA residues A2058, A2059, A2062, A2503, G2505, and G2611 (here and throughout the E. coli numbering) (Tu et al., 2005). One of the main mechanisms of resistance to macrolide antibiotics is based on dimethylation of A2058 by methyltransferase encoded in erm genes (Weisblum, 1995). Erm-catalyzed dimethylation of A2058 leads to a steric clash with the drug and reduces affinity of erythromycin for the ribosome. Similar to several other antibiotic resistance genes, erm genes are often inducible by erythromycin and similar drugs.

In an effort to combat resistance, a newer class of macrolides, known as ketolides, was developed (Bryskier, 2000). Ketolides show improved activity against strains with inducible erm genes and are believed to exhibit a tighter binding to the ribosome compared with macrolides of the previous generations (Poehlsgaard and Douthwaite, 2003; Poehlsgaard et al., 2005). In ketolides, cladinose is replaced by a keto function (hence the name of the class) and an extended alkyl-aryl side chain is attached at C11, C12 carbon atoms (in the ketolides prototype telithromycin) or at other positions of the lactone ring (Figure 4-1). Early biochemical and genetic studies showed that the extended side chain of ketolides establishes important new interactions with the ribosome that might account for increased efficacy of these drugs. Specifically, chemical probing and resistance mutations pointed to interactions of the 11,12 side chain of telithromycin with the rRNA residues in the loop of helix 35 of the E. coli 23S RNA and with U2609 (Garza-Ramos et al., 2001; Hansen et al., 1999; Xiong et al., 1999). However, subsequent crystallographic studies of the first clinically approved ketolide telithromycin bound to the bacterial (Deinococcus radiodurans) or archaeal (Haloarcula marismortui) ribosome showed the placement of the 11,12 side chain in a position that was hardly compatible with rRNA protections and mutations observed in the E. coli ribosome (Berisio et al., 2003; Tu et al., 2005). Furthermore, orientation of the alkyl-aryl side chain differed significantly between the reported D. radiodurans and H. marismortui structures, conforming to the general notion of idiosyncratic interactions of antibiotics with ribosomes of
various bacterial species (Sander et al., 2002; Wilson et al., 2005) and therefore leaving open the question of how the drug may bind to the ribosomes of pathogenic bacteria targeted by ketolides antibiotics.

Although telithromycin, the first ketolide introduced into medical practice in 2001, showed excellent activity against many macrolide resistant strains of Gram- positive pathogens, the safety issues that became apparent upon the wider use of the drug have curbed its clinical use (reviewed in (Rafie et al.)). The adverse effects associated with telithromycin spurred a search for newer ketolides. One of the novel promising drugs of this class is CEM-101 (Figure 4-1). The structure of CEM-101 is similar to that of telithromycin except for the chemical nature of the alkyl-aryl side chain (which in CEM-101 is represented by 11,12-carbamate-butyl-[1,2,3]-triazolyl- aminophenyl) and the presence of a fluorine atom linked to C2 of the lactone.

In microbiological tests involving a number of clinical pathogens, CEM-101 is characterized by lower minimal inhibitory concentration (MIC) values compared with telithromycin and exhibited enhanced activity against telithromycin-intermediate and telithromycin-resistant organisms (McGhee et al.). Furthermore, in comparison with telithromycin and cladinose-containing macrolides CEM-101 shows significantly enhanced accumulation in macrophages compared, which adds to its attractiveness from the medical standpoint.

Despite its favorable pharmacological properties, little is known about the mode of action and site of binding of CEM-101. In the following pages, biochemical and crystallographic approaches to characterizing the mode of action and site of binding of CEM-101, a new ketolide antibiotic, are reported.

4.3 Results and Discussion

4.3.1 Competition Binding Experiments Show CEM-101 Has the Same Binding Site as Previous Ketolides

Binding of CEM-101 to wild-type ribosomes from Gram-negative and Gram- positive bacteria was initially analyzed by competition with [14C] erythromycin. For that, we first analyzed binding of radiolabeled erythromycin to our preparations of E. coli and S. aureus 70S ribosomes. In saturation binding experiments, [14C] erythromycin readily bound to ribosomes from both bacteria, exhibiting $K_d$ of 66 ± 11 nM and 11 ± 1 nM for the E. coli and S. aureus ribosomes, respectively (data not shown). These values were comparable to those previously published (10^{-8}-10^{-7}M) (Douthwaite and Aagaard, 1993; Karahalios et al., 2006). Binding of erythromycin saturated close to 1 pmol of the drug per 1 pmol of E. coli or S. aureus ribosomes, indicating that the majority of the ribosomes in the preparation were competent for binding.

In competition binding experiments, CEM-101 readily displaced erythromycin from both types of ribosomes with IC50 of 155 ± 8 nM for the E. coli ribosome, and 117 ± 3 nM for the S. aureus ribosome (Figure 4-2 A, B) resulting in CEM-101 $K_d$ of 62 ± 3 nM (E. coli) and 12 ± 1 nM (S. aureus) (Table 4-1). In
parallel experiments, another ketolide, telithromycin, as well as cladinose-containing azithromycin exhibited a comparable affinity (Table 4-1). When radiolabeled \[^{14}\text{C}\] CEM-101 became available to us, its affinity for \textit{S. aureus} wild-type ribosomes was re-examined by saturation binding experiments (Figure 4-2C). A \(K_d\) of 50 ± 13 nM obtained with this approach was similar to that obtained by competition with erythromycin. Altogether, drug binding studies demonstrated that the new ketolide CEM-101 interacts with the ribosomal site that either coincides or overlaps with that of erythromycin and demonstrated that the drug binds to ribosomes of Gram-positive and Gram-negative bacteria with affinities similar to those of other macrolides.

4.3.2 The Alkyl Aryl Heterocycle of CEM-101 Has More Stable Interactions with rRNA than Telithromycin

The chemical structure of CEM-101 is different from the structures of conventional macrolides (Figure 4-1). It also shows important variation with the structure of telithromycin: the imidazolyl-pyridine moiety of the telithromycin 11,12 side chain is replaced with a triazolyl-aminophenyl conjugate in CEM-101. Therefore, although binding of telithromycin was previously studied by X-ray crystallography (Berisio et al., 2003; Dunkle et al.; Tu et al., 2005), it was unclear how closely binding of CEM-101 to the bacterial ribosome would match that of telithromycin. This consideration prompted us to characterize binding of CEM-101 to the \textit{E. coli} ribosome using crystallographic analysis.

We succeeded in obtaining the high-resolution structure of the \textit{E. coli} ribosome with the bound CEM-101 (Table 4-2). The general pose of CEM-101 in the ribosome is similar to that seen for telithromycin bound to the ribosome of \textit{E. coli} (Dunkle et al. 2010) (Figure 4-3A). The placement and configuration of the lactone ring and desosamine sugar of the two drugs are essentially indistinguishable. The triazole-aminophenyl head of the 11,12 side chain of CEM-101 makes a similar stacking interaction with the A752-U2609 base pair as the imidazole-pyridine moiety of telithromycin; it is located at a distance of 3.5 Å from the A752 and U2609 bases and oriented parallel to them. Importantly, the interactions of the CEM-101 (and telithromycin) alkyl-aryl side chains we observed in the \textit{E. coli} ribosome are principally different from those seen previously in crystallographic complexes of ketolides with the ribosomes of \textit{D. radiodurans} or \textit{H. marismortui} (Berisio et al., 2003; Schlunzen et al., 2003). We believe that the presence of the A752-U2609 base pair in the ribosomes of \textit{E. coli} and many pathogenic bacteria may account for this specific mode of interaction of the ketolides’ side chain. Because of the 23S rRNA sequence differences, formation of such a base pair is impossible in either \textit{D. radiodurans} or \textit{H. marismortui}. This consideration makes us believe that the structures of ketolides complexed with the \textit{E. coli} ribosome more accurately reflect interactions of the drugs with the ribosomes of pathogenic bacteria. Various ketolides differ from CEM-101 in the chemical nature of the alkyl-aryl side chain and the site of its attachment to the lactone scaffold. Nevertheless, in the RNA probing
experiments, all these drugs afford a near complete protection of A752 in the *E. coli* ribosome from chemical modification (Douthwaite et al., 2000; Hansen et al., 1999; Xiong et al., 2005; Xiong et al., 1999) indicating that the interaction of the alkyl-aryl side chain with the A752-U2609 base pair is important for binding of a range of clinically-relevant ketolides to the ribosome.

Despite a generally similar orientation of the side chains of CEM-101 and telithromycin, the variation in their chemical structures produces an important difference in the mode of binding. The atomic displacement parameter (ADP) refinement for the CEM-101 atoms shows that the extended arm of CEM-101 exhibits a significantly lower ADP value compared with the side chain of telithromycin (Figure 4-3B), whereas the average ADP values for all the other antibiotic atoms are very similar in both cases. This difference, which reflects better anchoring of CEM-101 in its binding site in the ribosome, likely results from additional hydrogen bonding interactions of the exocyclic amino group of aminophenyl in the side chain of CEM-101. Specifically, the amino group appears to serve as a H-bond donor to O4’ of A752 and O6 of G748, while it is an H-bond acceptor from N1 of G748. None of these interactions is possible for telithromycin.

### 4.3.3 Any Modifications Larger Than a Fluorine at the C2 Position of CEM-101 Will Lead to Steric Clashes with rRNA

A distinctive feature of CEM-101 compared to telithromycin and several other ketolides is the presence of a fluorine atom at the C2 position of the lactone ring. In the structure of CEM-101 bound to the *E. coli* ribosome, the fluorine atom is positioned near the glycosidic bond (atom N1) of C2611 and thus, can potentially contribute to the drug binding (Figure 4-3D). Although some reports questioned the importance of 2-F for the activity of ketolides (Keyes et al., 2003), comparison of the MIC values of CEM-101 with those of its analog that lacked the fluorine atom showed that CEM-101 more readily inhibited growth of *streptococci* carrying the erm methyltransferase gene (Table 4-3). Thus, fluorination of the C2 carbon atom may specifically contribute to a tighter binding of the drug to the ribosome dimethylated at A2058. In the structure of CEM-101 complexed to the *E. coli* ribosome, fluorine atom is only 2.7 Å away from N1 of C2611, indicating that any modifications larger than fluorine at this position would lead to a structural clash with rRNA.

### 4.4 Materials and Methods

#### 4.4.1 Introduction of Antibiotics Into the Crystals

Ribosomes were purified from MRE600 *E. coli* cells as described previously (Schuwirth et al., 2005). Ribosome crystals were grown and handled as described (Zhang et al., 2009), except that cryoprotection buffer was supplemented with 50μM CEM-101. The crystals were soaked in cryoprotection buffer plus CEM-101 for 12 to 24 hours, then flash frozen with liquid nitrogen.
4.4.2 Diffraction Data Collection, Refinement and Model Building
X-ray diffraction data were collected at beamline 12.3.1 of the Advanced Light Source, Lawrence Berkeley National Laboratory, using 0.1°-0.3° oscillations at 100K and recorded on an ADSC Q315 detector. X-ray diffraction data were reduced and scaled using HKL2000 (Otwinowski, 1998). The coordinates reported in 3I1M, 3I1N, 3I1O, and 3I1P were refined against the reflection data using the PHENIX software suite (Adams et al., 2010). \( F_o - F_c \) maps were calculated using PHENIX, and coordinates for CEM-101 were placed into this unbiased difference density using the software Coot (Emsley and Cowtan, 2004). Individual atomic displacement parameter values for the comparison between CEM-101 and telithromycin were calculated using PHENIX. Figures were made using PyMol.

4.4.3 Competition Experiments
Binding of erythromycin to *E. coli* and *S. aureus* ribosomes and competition experiments were done by size-exclusion chromatography using Bio-Gel P30 spin columns as described in (Xiong et al., 2005). Direct antibiotic binding experiments were done by incubating ribosomes at a 100nM concentration with varying concentrations of radiolabeled drug in a total volume of 160 μL in buffer A (20mM Tris-HCl [pH 7.5], 10mM MgCl₂, 150mM NH₄Cl, 6mM 2-mercaptoethanol) at 37°C for 15 min, then at 20°C for 10 min. The reactions were loaded onto the spin columns and centrifuged in a swinging-bucket microcentrifuge rotor for min at 1,000g at room temperature. The flow-through solution was collected; 130 μL was mixed with 5 mL of the scintillation cocktail. Radioactivity was measured in a scintillation counter and used to calculate the amount of erythromycin bound to ribosomes. For competition binding experiments, ribosomes (100 nM) were preincubated with 100 nM \(^{14}\)C erythromycin (48.8 Ci/mol; PerkinElmer) in 160 μL of buffer A at 37°C for 15 min, then at 20°C for 10 min. Competing antibiotics were added at varying concentrations and the binding mixture was incubated at 20°C for 100 min. The reactions were loaded onto the spin columns and the amount of ribosome-associated radioactivity was measured as described above. Binding data were analyzed using Prism software (GraphPad).

5. Summary and Conclusions

5.1 Conclusions Regarding Structures of the Ribosome in Intermediate States of Ratcheting


In conclusion, I have shown that x-ray crystal structures of the ribosome in an intermediate ratcheted state revealed that the ribosome adopts the ratcheted
conformation through a series of stepwise intermediates. This rationalized single molecule FRET data showing entry of tRNA into the P/E hybrid state of binding occurs at a faster rate than intersubunit rotation (Munro et al., 2007; Cornish et al., 2008). The structures showed that the partial rotation and swiveling of the 30S head domain allows tRNA to be positioned into the A/P, P/E hybrid state of binding before full rotation of the 30S body and platform domains has occurred. This result was recently confirmed by a time resolved cryo-electron microscopy experiment (Fischer et al., 2010). The intermediate ratcheted structure also revealed the positions that the intersubunit bridges adopt during their conversion to the ratcheted conformation, and in particular, revealed the malleability of the contacts between the proteins L5 and S13, where an approximately 20 Å movement of S13 takes place relative to L5. The fact that S13 is tuned to facilitate ribosome dynamics was suggested by previous genetic and biochemical experiments (Cukras and Green, 2005).

While the x-ray structures described suggest at least two macrostates, R2 and RF occur during the ratcheting and tRNA translocation process, the number of degrees of freedom in the ribosome suggests that the number of conformations that could contribute to the process is large. First, as part of mRNA decoding, the shoulder domain of the 30S subunit has been imaged in two different conformations, more or less open relative to the subunit interface region (Figure 5-1) (Ogle et al., 2002). Second, the 30S subunit can adopt four different rotational states relative to the 50S subunit (Figure 5-2) (Zhang et al., 2009). Third, the head domain has been seen in five different rotational states relative to the 30S subunit body and platform (Figure 5-2) (Agirrezabala et al., 2008; Allen et al., 2005; Berk et al., 2006; Chandramouli et al., 2008; Connell et al., 2008; Laurberg et al., 2008; Schuwirth et al., 2005; Selmer et al., 2006; Spahn et al., 2004; Taylor et al., 2007; Zhang et al., 2009). These three degrees of freedom seem to be decoupled within the apo-70S ribosome (Schuwirth et al., 2005; Zhang et al., 2009). The total configurational space of large-scale motions in the 30S subunit includes 40 states from known structures. Including the changes in the 50S subunit L1 region or L7/12 region and any local conformational changes, expands the accessible configurations even more. The partitioning of the 30S and 50S subunits in intact ribosomes among these states is intimately related to a given step in translation because of the translation factors and ligands bound in each step. Future work is needed to sort out the energy landscape of these global conformations in translocation and, in particular, how EF-G influences translocation to promote the forward direction of mRNA and tRNA movement (Munro et al., 2009).

5.2 Conclusions Regarding Structures of the Ribosome with Antibiotics Bound in or near the Peptidyl Transferase Center

The structures of the ribosome bound to antibiotics presented here along with the methods reported and cited, show E. coli ribosome crystals are a tractable tool for analysis of the details of antibiotic binding to the ribosome and
mechanisms of inhibition. Specifically, I have shown that because the *E. coli* ribosome has higher sequence homology to bacterial species of medical interest, such as *S. aureus* (Figure 3-1C, 3-3C and Table 3-1) it provides more accurate information concerning how antibiotics interact with the rRNA of medically relevant organisms. The structures presented here, addressed and resolved lingering discrepancies between the data for antibiotics bound to ribosomal subunits from *H. marismortui* and *D. radiodurens*. The structures confirm that the mechanism of protein synthesis inhibition for clindamycin and chloramphenicol involves interference with correct positioning of aminoacyl tRNA in the A site. Also the data show that the ketolides telithromycin and CEM-101 interact with the rRNA of medically relevant bacterial species differently than that of archaeal or divergent eubacterial species, due to sequence differences at C2055, A752, U2609 and A2058 (Figure 3-1C, 3-3C and Table 3-1). Experiments with CEM-101, show that analysis of new protein synthesis inhibitors can be performed by x-ray crystallography using *E. coli* ribosomes, while the compounds are still in development. This could increase the efficiency of new antibiotic discovery.

5.3 Future Prospects and Challenges for Mechanistic Studies of Protein Synthesis

Two challenges are clear for advancing mechanistic studies of protein synthesis. One was mentioned previously: current structural data has shown that the domains of the 30S subunit and the L1 and L7/12 regions of the large subunit can adopt many conformations, which seem to be decoupled from one another, suggesting many conformations of the ribosome may exist that have yet to be observed. A second even more significant problem is that "the proliferation of [ribosome] structures has led to a proliferation of models, which need to be tested" (Noller, 2010). The problem has arisen because x-ray crystal structures provide the basis for proposing very detailed mechanistic models, but there is a lack of complimentary techniques with which to test the hypotheses in sufficient detail. However clever uses of existing techniques could overcome the problem. Following are two examples of experiments which fulfill the criteria of complementarity to x-ray crystallography and sufficiency of detail.

The idea that ratcheting or a conformational change of the 30S subunit was coupled to, and responsible for, tRNA translocation was first proposed on theoretical grounds by Bretscher (Bretscher, 1968). In the ensuing years, particularly once cryo-electron microscopy data became available for the ratcheted conformation of the ribosome with a hybrid state tRNA bound (Valle et al., 2003a), it was believed that ratcheting of the ribosome was necessary for tRNA translocation, yet this had not been directly tested. An experiment by Horan and Noller sought to address this (Horan and Noller, 2007). To control the conformation of the ribosome, mutant *E. coli* ribosomes were constructed which carried cysteines at S6 residue 81 and L2 residue 123, two residues one from each ribosomal subunit, which lie adjacent to each other on the intersubunit interface in the un-ratcheted conformation, but are distant from each other in the
ratcheted conformation of the ribosome. The presence or absence of a disulfide bond between the residues could be controlled by adding or removing a reducing agent. Since EF-G catalyzed translocation of tRNA is insensitive to the differences in reducing agent, the translocation reaction could be assayed in either condition, with the result that translocation was always very incomplete in an oxidizing environment, whereas in a reducing environment in which the ribosome can ratchet, translocation proceeded normally. Thus structural data was able to inform the design of an experiment to directly test the hypothesis that ratcheting is necessary for translocation.

An experiment by Fischer, Stark and colleagues provided a similarly unique insight into protein synthesis mechanisms using time resolved cryo-electron microscopy (Fischer et al., 2010). Fischer took advantage of the fact that tRNA translocation can happen in the reverse direction, as well as, the forward direction (Shoji et al., 2006), but is much slower in reverse. Reverse translocation is necessary to correct errors in translocation and possibly also for extrusion of secreted proteins through the translocon. By spotting a reverse translocation reaction onto grids at several timepoints over the course of 20 minutes and freezing the grids, Fischer generated distributions of translocation intermediates on each grid. The particles could then be classified and sorted, becoming the basis for particle reconstructions of major and minor conformational populations present at each time point. The time resolution of the experiment, along with analysis of the similarities and differences between the populations, allowed the individual particle reconstructions to be assembled into a plausible pathway showing conformational changes as translocation of tRNA occurs. The model can be viewed online as a movie at http://www.nature.com/nature/journal/v466/n7304/extref/nature09206-s3.mpg.

The continued contribution of x-ray crystallography to resolve our incomplete structural knowledge of the ribosome, along with methods such as those described above to test detailed mechanistic models, should allow continued increase in our understanding of the translation of the genetic code.
Citations


antibiotics with the peptidyl transferase centre in eubacteria. Nature 413, 814-821.


**Figure 1-1. Major features of the structure of the E. coli ribosome.** (A) View of the 70S ribosome from the solvent side of the 30S subunit. Structural landmarks are marked: Head, Body, Platform, Spur in the 30S subunit; L1 and L11 arms and Central Protuberance (CP) in the 50S subunit. In the 30S subunit, 16S rRNA (light blue) and small subunit (S) proteins (dark blue) are shown. In the 50S subunit, 5S and 23S rRNA (grey) and large subunit (L) proteins (purple) are shown. Proteins L1 and L7/L12 are modeled onto the structure of the E. coli 50S subunit by superposition of homologous structures (Selmer et al., 2006; Diaconu et al., 2005), but are not visible in x-ray crystal structures of the E. coli ribosome due to dynamics in them. (B) View of the 70S ribosome from the perspective of the 50S subunit. Labeling as in panel A. (C) View of the interface between the ribosomal subunits. Contacts, or “bridges” to the opposite subunit are labeled and colored red.
Figure 1-2. Binding sites for tRNA in the ribosome. (A) Schematic of the 70S ribosome indicating the positions of the aminoacyl-tRNA, peptidyl-tRNA, and exit-tRNA binding sites (A, P, and E sites, respectively). Additional binding sites for tRNAs occur for initiator-tRNA (I on the 50S subunit), during mRNA decoding (T on the 50S subunit), and during translocation (Allen et al., 2005; Valle et al., 2003a). (B) View of the structure of the 70S ribosome rotated 90° about the vertical axis in Figure 1-1A. The binding sites for A-site tRNA and mRNA are marked. (C) Slice through the 70S ribosome as indicated by icon. Views of the ribosome with tRNAs bound in the A/A, P/P and E/E sites (30S/50S sites, respectively). The approximate locations of the I, and T sites on the 50S subunit are marked. Protein L1, the Central Protuberance, and the L11 arm are marked.
Figure 1-3. Structural features of the 30S ribosomal subunit. (A) Global features of the 30S subunit viewed from the perspective of the interface with the 50S subunit. The three tRNA binding sites are labeled A, P, and E. The anticodon stem-loops of the A-site (yellow), P-site (orange), and E-site (red) tRNAs are shown for perspective. Color coding of the 30S subunit is the same as in Figure 1-1. (B) Close-up of the mRNA decoding site with the nucleotides that read out the mRNA codon/tRNA anticodon helix minor groove indicated. Messenger RNA is in green. (C) View of A-site tRNA in the process of mRNA decoding, as viewed by cryo-EM. The tRNA is in the A/T state at this step of decoding. The GTPase Associated Center (GAC) of the ribosome is shown.
Figure 1-4. Conformational changes in the 30S subunit associated with mRNA and tRNA translocation. (A) Rotation of the 30S subunit relative to the 50S subunit. The axis of rotation is approximately perpendicular to the page. (B) Positioning of tRNAs in hybrid states of binding when the 30S subunit is rotated relative to the 50S subunit. Prior to subunit rotation, tRNAs occupy the A/A and P/P sites (Figure 1-2C), and shift into the A/P and P/E sites after subunit rotation. (C) Movement of the 30S subunit head domain during translocation, viewed from the perspective of the 30S subunit interface with the 50S subunit. Proteins S19, S13 and h33 of 16S rRNA are marked.
Figure 1-5. The GTPase center of the 50S subunit. A view of interactions made between EF-G and the ribosome is pictured. A model of proteins L7/12 docked onto the 50S using electron density derived from EM maps is shown (Diaconu et al., 2005). Proteins L7/12 are dynamic and are not clearly visible in x-ray crystal structures of the intact ribosome. The Sarcin-Ricin Loop (SRL) (red) is shown contacting the G-domain (green) of EF-G. The GTPase domain of other translation factors likely occupy similar positions to that of EF-G.
Figure 1-6. Dynamics of the L1 arm. View of the L1 arm in the 50S subunit from the perspective of the 30S subunit. E-site tRNA is shown for reference. The L1 arm is composed of the L1 protein and 23S rRNA helices 76-78.
Figure 1-7. Path of mRNA into and out of the ribosome.  

(A) The region of mRNA 5’ of the tRNA binding sites (green) can base pair with the 3’ end of 16S rRNA (light blue) during initiation.  

(B) The 3’ region of mRNA (green) following the A-site codon enters through a path surrounded by ribosomal proteins S3 (gold), S4 (red) and S5 (orange).
Figure 1-8. Interactions of acceptor ends of tRNAs with 23S rRNA in the 50S subunit. (A) Binding of tRNAs in the P site and A site in the peptidyl transferase center (PTC). tRNAs are pictured charged with an amino acid on the terminal adenosine of each tRNA. Hydrogen-bonds between tRNAs and 23S rRNA are shown as black dashes. An arrow indicates the nucleophilic amine group which can attack the ester bond linking the nascent peptide chain to P-site tRNA. (B) Binding pocket in the 50S subunit for the 3'-Adenosine of E-site tRNA. Hydrogen-bonds between E-site tRNA and 23S rRNA are shown a black dashes. Note that the pocket differs slightly in archaeal and eukaryotic ribosomes (Selmer et al., 2006).
Figure 1-9. Example Images of Ribosome Crystals and the Resulting X-Ray Diffraction Pattern. A magnified view of typical ribosome crystals is shown on the left, with a diffraction pattern typical of a well diffracting crystal on the right.
Figure 2-1. Rotated states of the ribosome.

(A) View of the bacterial 70S ribosome, composed of the small (30S) ribosomal subunit and the large (50S) ribosomal subunit. The small subunit of the ribosome (blue) can rotate from a starting conformation seen in post-initiation and termination states (Berk et al., 2006) (state $R_0$, black outline) to a fully rotated conformation seen in elongation, termination and recycling steps of translation (state $R_F$, red outline) (Connell et al., 2007). 30S features include: Head, Platform, Body. The 50S subunit is shown in grey. Letters indicate the positions of the aminoacyl (A), peptidyl (P), and exit (E) tRNA binding sites. 

(B) Schematic of tRNA binding states on the ribosome. In the transition of the ribosome to the fully rotated state, tRNAs shift from binding in the A/A and P/P sites (30S subunit/50S subunit, respectively) to occupy hybrid binding sites (A/P and P/E for 30S/50S sites). The view of the ribosome is rotated 90° from that in A.

(C) Rotations of the head domain of the small ribosomal subunit. Letters indicate the locations of the aminoacyl (A), peptidyl (P), and exit (E) tRNA binding sites on the large subunit. In state $R_0$ (black), the head domain is centered over the P site (~0° rotation). Rotations of the head domain towards the E site of up to 14° (red) have been observed (Spahn et al., 2004). The 5’ to 3’ direction of mRNA, which threads around the neck region of the 30S subunit, is also indicated.
Figure 2-2. Structure of the apo-70S ribosome in an intermediate state of intersubunit rotation, state R$_2$. (A) A view of the ribosome with features of the 30S subunit and 50S subunit labeled. (B) Comparison of the ribosome in state R$_2$ with the ribosome in state R$_0$ (Berk et al., 2006; Zhang et al., 2009), with the 50S subunit serving as reference. Arrows indicate the direction of movement in the transition from state R$_0$ to state R$_2$. The distance changes in 30S subunit positions are color-coded in Å units, as shown, in this and the subsequent panels. Ribosomal RNA and proteins in the 50S subunit are colored grey and magenta, respectively. 30S features include: Head, Neck, Platform, Body, Shoulder, and Spur. 50S features include: L$_1$, protein L1/rRNA arm; CP, central protuberance; L$_{11}$, protein L11/rRNA arm, L$_9$, protein L9. The approximate location of proteins L$_7$/L$_{12}$ and L$_1$, not observed in the structure, are noted in grey. (C) Comparison of the ribosome in state R$_2$ to the ribosome in state R$_0$, viewed from the perspective of the 50S subunit. Difference vectors between phosphorous and Ca atoms are shown to the right, with arrows indicating the direction of the change. (D) Comparison of the ribosome in state R$_2$ to the ribosome in state R$_1$ (Selmer et al., 2006). (E) Comparison of the ribosome in state R$_2$ to the ribosome in the fully rotated state R$_F$ (Connell et al., 2007).
Figure 2-3. Contacts, or “bridges”, between the ribosomal subunits in the apo-70S ribosome in state \( R_2 \). (A) The position of bridges in state \( R_2 \) compared to those in state \( R_0 \). Bridge numbering is the same as in (Schuwirth et al., 2005). The direction of view and color coding are the same as in Fig. 2-2C. Bridge B1a (asterisk), includes the A-site Finger (H38 in 23S rRNA) which spans the subunit interface parallel to the A and P sites (Connell et al., 2007). This contact is not visible in the present structures due to disorder at the end of H38 in both states of the ribosome. (B) Bend in rRNA helix h44 in 16S rRNA that accommodates rotated state \( R_2 \). Nucleotides and 16S rRNA helices are marked. The view is the same as in Fig. 2-2C. (C) Bridge B7a in state \( R_2 \) compared to that in states \( R_0 \) and \( R_1 \). Nucleotide A702 in 16S rRNA in the 30S subunit (light blue) and nucleotides in H68 of 23S rRNA in the 50S subunit (grey) are shown for state \( R_2 \). Nucleotide A702 in state \( R_0 \) or \( R_1 \) is shown in red. The N1 position of A702 that would be methylated by dimethylsulfate is marked (Moazed et al., 1989b).
Figure 2-4. Changes in the position of the head domain in the 30S subunit in state $R_2$.  
(A) Bridge B1 in ribosomes in state $R_1$. The tRNAs bound in the 30S subunit A site (yellow), and in the P/P (orange) and E/E sites (red) are shown. Domains in protein S13 in the 30S subunit head domain (blue) and protein L5 in the 50S subunit (purple) are marked. An asterisk marks the approximate location of the A-site finger (ASF) helix H38 in 23S rRNA, the tip of which is disordered in the crystal structure. Protein L31, not seen in E. coli 70S ribosome structures, has been removed for clarity.

(B) Bridge B1 in the apo-70S ribosome in state $R_2$ (light blue) compared to state $R_F$ (red). Domains in protein S13 in the 30S subunit head domain and protein L5 in the 50S subunit are marked. Asterisk as in panel A.

(C) Position of full-length tRNAs modeled onto the apo-70S ribosome in state $R_2$. The superposition used the head domain of the 30S subunit in the fully-rotated state $R_F$ (Aggirezabala et al., 2008) for reference. Surfaces of the modeled tRNAs (yellow and orange) are compared to the position of tRNAs in state $R_1$, as described in panel A and shown as ribbons.

(D) Position of full-length tRNA in the P site of state $R_1$ modeled onto the ribosome complexed with ASL$^{Met}$ in the P site in state $R_2$, using the 30S subunit body and platform of the ribosome in state $R_1$ as a reference. Surface of the modeled tRNA (blue) is compared to the position of the P-site ASL$^{Met}$ in state $R_2$ (blue) and tRNAs in state $R_1$ (described above) shown as ribbons.

(E) Step-wise rearrangements in the ribosome along the ratcheting pathway. The molecular envelope of the 30S subunit is shown for clarity. Domains of the 30S subunit (head, body, platform), tRNA binding sites (A, P, and E, respectively), and bridges B1b, B2a, and B3 are shown. The view is the same as in Fig. 2B. Arrows indicate the direction of movement from one state to the next.
Figure 3-1 Binding sites for antibiotics in the PTC and peptide exit tunnel. (A) The chemical structures of erythromycin (a macroline), clindamycin (a lincosamide), telithromycin (a ketolide), and chloramphenicol (a phenicol) are shown as stick models within the 50S subunit. Erythromycin (green), telithromycin (pink), clindamycin (purple) and chloramphenicol (orange) are shown as stick models. Ribbons denote the sugar phosphate backbone of 23S rRNA (grey) with nucleotides of interest colored light blue, the acceptor ends of A-site tRNA (yellow) and P-site tRNA (red). The location of the peptide exit tunnel is labeled “exit” and an icon indicating the point of view is shown on the left. (B) An overview of the antibiotic binding sites on the 23S rRNA backbone is shown. (C) The secondary structure of domain V of 23S rRNA showing elements of the PTC and the adjacent peptide exit tunnel. A detailed view (top) is shown of the central region of domain V outlined in a red box (bottom). Nucleotides which are divergent between *E. coli* and *H. marismortui* are shown in red. *D. radiodurans* diverges from *E. coli* at the 2057-2611 base pair and at nucleotides 752 and 2556. Ribosomal RNA helices emanating from this region are marked with dotted lines.
Figure 3-2. Erythromycin bound to the *E. coli* ribosome.

(A) Unbiased difference (*F*$_{obs}$–*F*$_{calc}$) electron density for erythromycin bound to the *E. coli* ribosome contoured at 3.5 standard deviations from the mean.

(B) A comparison of erythromycin bound to the *H. marismortui* 50S subunit (yellow) or *E. coli* ribosome (green). 23S rRNA for the *E. coli* ribosome is shown in grey. Nucleotide positions at which mutation confers resistance for erythromycin are labeled in red.
Figure 3-3. Telithromycin bound to the *E. coli* ribosome.

(A) A comparison of the conformations reported for telithromycin bound to the ribosome. 23S rRNA for *E. coli* is shown in grey. Telithromycin models from *H. marismortui* (gold), *D. radiodurans* (cyan) and *E. coli* (pink) are shown. (B) Difference electron density ($F_o$-$F_c$) for telithromycin contoured at 3 standard deviations from the mean. (C) A sequence alignment of 23S rRNA for species from which ribosome or 50S subunit crystal structures are available (*H. marismortui*, *D. radiodurans*, *T. thermophilus* and *E. coli*) along with species of medical interest (*S. aureus* and *S. pneumoniae*). Nucleotides A752-U2609 (*E. coli* numbering) form a base pair on which the alkyl aryl arm of telithromycin stacks.
Figure 3-4 Footprinting experiments show telithromycin protects A752 in *E. coli* and *S. aureus*. Interactions of antibiotics erythromycin (E) or telithromycin (T) in solution with ribosomes prepared from archaea (H. halobilum) or three different bacterial species (*D. radiodurans*, *E. coli* or *S. aureus*) as revealed by dimethylsulfate (DMS) probing. Upper panel, protections of A2058 and A2059 by both antibiotics to all the tested ribosomes. Lower panel, protection of A752 from DMS modification by telithromycin in *E. coli* or *S. aureus* ribosomes, but not in either *H. halobilum* or *D. radiodurans* ribosomes. Lanes on the gels are labeled as following: A, an A-specific sequencing reaction; K, unmodified ribosome; 0, ribosome modified with DMS in the absence of antibiotics; E and T, ribosome modified with DMS after preincubation with 50 μM of erythromycin or telithromycin, respectively. *Data courtesy of Liqun Xiong.*
Figure 3-5. Clindamycin bound to the *E. coli* ribosome.

(A) The conformations of clindamycin bound to the *D. radiodurans* 50S subunit has the pyrrolydinylyl propyl group rotated by 180 degrees. (B) *E. coli* 23S rRNA is superpositioned with *H. marismortui* 23S rRNA to reveal that some nucleotides (2504-2507) are shifted in space due to the sequence difference at 2055, C in *E. coli*, A in *H. marismortui*. (C) The van der Waals radii of atoms in nucleotides C2452 and U2506 which pack tightly against the propyl group of clindamycin are shown as spheres. Carbon atoms in nucleotides are grey spheres, while carbon atoms in clindamycin are magenta spheres. The chlorine atom in clindamycin is shown as a green sphere. (D) A diagram of the interactions of clindamycin with the *H. marismortui* (right) and *E. coli* (left) ribosomes. Dashed lines represent hydrogen bonds.
Figure 3-6. Chloramphenicol bound to the *E. coli* ribosome.

(A) Unbiased difference electron density \((F_{\text{obs}} - F_{\text{calc}})\) contoured at 3.5 standard deviations from the mean for chloramphenicol bound to the *E. coli* ribosome (left). At right, the structure of chloramphenicol in the context of *E. coli* rRNA is shown (orange) compared to the structure of chloramphenicol reported bound to *D. radiodurans* ribosomal subunits (cyan).

(B) Chloramphenicol (orange) bound to the A-site cleft of the *E. coli* ribosome. Hydrogen bonds are shown as black dashes. Nucleotides that are sites of resistance mutations to chloramphenicol are labeled in red.
Figure 4-1. The chemical structures of erythromycin, a macrolide and the ketolides telithromycin and CEM-101.
Figure 4-2. Binding of CEM-101 to wild type bacterial ribosomes. (A, B) Competition of CEM-101 with \[^{14}C\] erythromycin for binding to *E. coli* (A) or *S. aureus* (B) ribosomes. (C) Saturation binding of \[^{14}C\] CEM-101 to *S. aureus* ribosomes (*Data courtesy of B. Llano-Sotello*).
Figure 4-3. The structure of the *E. coli* ribosome bound to CEM-101. (A) The position of CEM-101 (pink) on the ribosome showing hydrogen bonds formed between the aniline moiety of CEM-101 and rRNA (gray). (B) Electron density (2Fo-Fc) within the CEM-101 binding pocket, contoured at 2 standard deviations above the mean mesh. (C) Difference in atomic displacement parameter (ADP) values for the 11,12 side chains of CEM-101 and telithromycin. A scale is shown (upper right) describing the values of the atom colors. (D) Proximity of the fluorine atom of CEM-101 to rRNA in the drug binding site. The distance between the fluorine of CEM-101 and N1 of C2611 of 23S rRNA is 2.7 Å.
Figure 5-1. Large scale motions in the ribosome during translocation. Arrows indicate features of the 30S subunit (intact subunit, head domain, body domain) and 50S subunit (protein L1 arm, protein L11 arm) that move during translocation. Dynamics within the L7/L12 proteins (15) are not shown. 30S subunit rRNA and proteins are in light blue and dark blue, respectively. 50S subunit rRNAs and proteins are in gray and magenta, respectively.
Figure 5-2. Intermediate states of subunit ratcheting. Stepwise rearrangements in the ribosome along the ratcheting pathway, viewed from the perspective of the 50S subunit. Arrows indicate the direction of movement from one state to the next. The changes in the distance of the 30S subunit positions are color-coded in Ångstrom units and are shown as difference vectors between corresponding RNA phosphorous or protein C-α atoms. Domains of the 30S subunit (head, body, and platform) are labeled.
**Table 2-1** Diffraction and refinement statistics for crystals of ribosomes in an intermediate rotated state.

<table>
<thead>
<tr>
<th></th>
<th>apo-70S</th>
<th>ASL&lt;sup&gt;Met&lt;/sup&gt;*</th>
<th>2 x ASL&lt;sup&gt;Phe&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P212121</td>
<td>P212121</td>
<td>P212121</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>211.9, 434.9, 622.9</td>
<td>210.7, 435.1, 628.7</td>
<td>211.0, 433.1, 624.5</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>40-3.2 (3.52-3.45)**</td>
<td>76-3.8 (4.28-4.09)</td>
<td>73-3.7 (4.27-4.07)</td>
</tr>
<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt; or R&lt;sub&gt;merge&lt;/sub&gt;</td>
<td>17.1(73.2)</td>
<td>11.1(38.1)</td>
<td>7.8(32.2)</td>
</tr>
<tr>
<td>I / σ(I)</td>
<td>6.9(2.0)</td>
<td>6.9(2.1)</td>
<td>7.9(2.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.2(94.5)</td>
<td>78.8(67.4)</td>
<td>75.9(64.0)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.7(3.6)</td>
<td>2.2(1.7)</td>
<td>2.2(1.4)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>40-3.2</td>
<td>76-3.8</td>
<td>73-3.7</td>
</tr>
<tr>
<td>No. reflections</td>
<td>904,039</td>
<td>438,242</td>
<td>452,724</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt; / R&lt;sub&gt;free&lt;/sub&gt;</td>
<td>19.5/25.2</td>
<td>20.7/25.3</td>
<td>22.7/26.8</td>
</tr>
<tr>
<td>No. atoms</td>
<td>284,560</td>
<td>285,465</td>
<td>286,195</td>
</tr>
<tr>
<td>R.m.s. deviations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.020</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.451</td>
<td>1.549</td>
<td>1.519</td>
</tr>
</tbody>
</table>

*Two crystals were used for this dataset. **Data beyond the high-resolution shell in parenthesis was used for refinement and map calculation, and extend to an I / σ(I) of about 1.
Table 3-1. Conservation of peptidyl transferase center and adjacent peptide exit tunnel nucleotides

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Eubacteria</th>
<th>% Conserved**</th>
<th>Archaea</th>
<th>% Conserved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position*</td>
<td>Nucleotide</td>
<td></td>
<td>Nucleotide</td>
<td></td>
</tr>
<tr>
<td>2055</td>
<td>C</td>
<td>100</td>
<td>A</td>
<td>88</td>
</tr>
<tr>
<td>2451</td>
<td>A</td>
<td>100</td>
<td>A</td>
<td>100</td>
</tr>
<tr>
<td>2452</td>
<td>C</td>
<td>100</td>
<td>C</td>
<td>86</td>
</tr>
<tr>
<td>2504</td>
<td>U</td>
<td>100</td>
<td>U</td>
<td>88</td>
</tr>
<tr>
<td>2505</td>
<td>G</td>
<td>100</td>
<td>G</td>
<td>99</td>
</tr>
<tr>
<td>2506</td>
<td>U</td>
<td>100</td>
<td>U</td>
<td>98</td>
</tr>
<tr>
<td>2507</td>
<td>C</td>
<td>100</td>
<td>C</td>
<td>99</td>
</tr>
</tbody>
</table>

*E. coli numbering
**The percent conservation is calculated from the seed sequence alignments for bacterial 23S rRNA and archaeal rRNA.
Table 3-2. Diffraction and refinement statistics for 70S ribosome complexes with antibiotics.

<table>
<thead>
<tr>
<th></th>
<th>Erythromycin</th>
<th>Telithromycin*</th>
<th>Clindamycin*</th>
<th>Chloramphenicol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁₂₁₂₁</td>
<td>P2₁₂₁₂₁</td>
<td>P2₁₂₁₂₁</td>
<td>P2₁₂₁₂₁</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) (Å)</td>
<td>211.9</td>
<td>210.7</td>
<td>211.0</td>
<td>211.4</td>
</tr>
<tr>
<td>(b)</td>
<td>434.5</td>
<td>433.2</td>
<td>434.4</td>
<td>434.0</td>
</tr>
<tr>
<td>(c)</td>
<td>623.5</td>
<td>618.8</td>
<td>618.9</td>
<td>621.2</td>
</tr>
<tr>
<td>(\alpha, \beta, \gamma) (°)</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>100-3.10</td>
<td>100-3.25</td>
<td>80.0-3.30</td>
<td>100-3.2</td>
</tr>
<tr>
<td>(R_{\text{sym}} \text{ or } R_{\text{merge}})</td>
<td>10.1 (75.3)</td>
<td>8.3 (45.5)</td>
<td>12.0 (74.3)</td>
<td>7.3 (52.7)</td>
</tr>
<tr>
<td>(I / \sigma(I))</td>
<td>7.9 (1.1)</td>
<td>9.4 (1.9)</td>
<td>12.5 (1.7)</td>
<td>9.6 (1.5)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>90.3 (78.2)</td>
<td>91.2 (81.1)</td>
<td>83.2 (70.6)</td>
<td>81.3 (66.9)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.3 (2.6)</td>
<td>2.5 (1.8)</td>
<td>6.3 (4.4)</td>
<td>1.9 (1.5)</td>
</tr>
</tbody>
</table>

| **Refinement**              |              |                |              |                 |
| Resolution (Å)              | 82.4-3.10    | 85.1-3.24      | 76.3-3.28    | 100-3.20        |
| No. reflections             | 860,649      | 754,750        | 661,143      | 759,197         |
| \(R_{\text{work}} / R_{\text{free}}\) (%) | 20.4/25.3 | 19.9/25.4 | 18.7/24.1 | 19.0/25.3 |
| No. atoms                   | 284,613      | 284,553        | 284,546      | 284,403         |
| R.m.s. deviations           |              |                |              |                 |
| Bond lengths (Å)            | 0.006        | 0.006          | 0.005        | 0.005           |
| Bond angles (°)             | 1.46         | 1.70           | 1.35         | 1.41            |

*Two crystals were used to measure each of these data sets.
†Values in parentheses are for the highest-resolution shell.
Table 4-1. Dissociation constants (Kₐ) of CEM-101 and other macrolides binding to *E. coli* or *S. aureus* ribosomes. *(Data courtesy of B. Llano-Sotello)*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycinᵃ</td>
<td>66 ± 11</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>CEM-101ᵃ</td>
<td>ND</td>
<td>50 ± 13</td>
</tr>
<tr>
<td>CEM-101ᵇ</td>
<td>62 ± 3</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Telithromycinᵇ</td>
<td>49 ± 2</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Azithromycinᵇ</td>
<td>28 ± 2</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

ᵃ Determined by measuring direct binding of radiolabeled compound.
ᵇ Determined by competition with radiolabeled erythromycin.
Table 4-2. Diffraction statistics for crystals of the *E. coli* 70S ribosome complexed with CEM-101.

<table>
<thead>
<tr>
<th><strong>Data collection</strong>*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space group</strong></td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td><strong>Cell dimensions</strong></td>
<td></td>
</tr>
<tr>
<td>( a ) (Å)</td>
<td>210.7</td>
</tr>
<tr>
<td>( b )</td>
<td>433.2</td>
</tr>
<tr>
<td>( c )</td>
<td>618.8</td>
</tr>
<tr>
<td>( \alpha, \beta, \gamma ) (°)</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td><strong>Resolution (Å)</strong></td>
<td>100-3.11</td>
</tr>
<tr>
<td></td>
<td>(3.17-3.11)†</td>
</tr>
<tr>
<td><strong>( R_{sym} ) or ( R_{merge} )</strong></td>
<td>11.1 (89.9)</td>
</tr>
<tr>
<td><strong>( I / \sigma(I) )</strong></td>
<td>8.27 (1.19)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>93.8 (85.3)</td>
</tr>
<tr>
<td><strong>Redundancy</strong></td>
<td>3.3 (2.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Refinement</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resolution (Å)</strong></td>
<td>69.7-3.10</td>
</tr>
<tr>
<td><strong>No. reflections</strong></td>
<td>821,883</td>
</tr>
<tr>
<td><strong>( R_{work} / R_{free} ) (%)</strong></td>
<td>0.22/0.26</td>
</tr>
<tr>
<td><strong>No. atoms</strong></td>
<td>284,555</td>
</tr>
<tr>
<td><strong>R.m.s. deviations</strong></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.006</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.452</td>
</tr>
</tbody>
</table>

* Two crystals were used.
† Values in parentheses are for highest-resolution shell.