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SPECIFICITY DETERMINANTS OF ArmA, A RIBOSOMAL RNA METHYLTRANSFERASE THAT CONFERS ANTIBIOTIC RESISTANCE

Tamara Zarubica
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SPECIFICITY DETERMINANTS OF ArmA, A RIBOSOMAL RNA METHYLTRANSFERASE THAT CONFERS ANTIBIOTIC RESISTANCE

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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<td>ArmA</td>
<td>Aminoglycoside resistance methyltransferase</td>
</tr>
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<td>βME</td>
<td>β- Mercaptoethanol</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IF-3</td>
<td>Initiation Factor 3</td>
</tr>
<tr>
<td>KsgA</td>
<td>Kasugamycin resistance methyltransferase</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
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<tr>
<td>MgOAC</td>
<td>magnesium acetate</td>
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<td>Rmt</td>
<td>ribosomal methyltransferase</td>
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<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
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<tr>
<td>Sgm</td>
<td>Sisomicin- gentamicin resistance methyltransferase</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
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Abstract

Specificity Determinants of ArmA, a Ribosomal RNA Methyltransferase, that confers antibiotic resistance

By Tamara Zarubica

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2010

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Associate Professor, Department of Physiology and Biophysics

Bacterial resistance to 4,6-type aminoglycoside antibiotics, which target the 30S ribosomal subunit, has been traced to the arm/rmt family of rRNA methyltransferases. These plasmid-encoded enzymes transfer a methyl group from S-adenosylmethionine to N7 of the buried G1405 in the aminoglycoside binding site of 16S rRNA in the 30S ribosomal subunit. Neither 16S rRNA alone nor intact 70S ribosome is an efficient substrate for armA methyltransferase. To more fully characterize this family of enzymes,
we have investigated the substrate requirements of ArmA. We determined the Mg\textsuperscript{2+} dependence of ArmA activity and found that the enzyme could recognize both translationally active and translationally inactive forms of 30S subunits.

To identify the site of interaction between ArmA and the 30S subunit, we used hydroxyl radical cleavage of 16S rRNA mediated by ferrous iron chelated to several sites on the ArmA molecule that were mutated to cysteine. This data suggests that significant conformational changes in 30S structure are involved in binding of ArmA. We hypothesized that a precursor intermediate in the biogenesis of the 30S subunit might be the optimal substrate for ArmA enzymes \textit{in vivo}. To test this, we prepared 30S particles partially depleted of proteins by treatment with increasing concentrations of LiCl and assayed them for ArmA methylation. Even low concentrations of LiCl alter the 30S particles and greatly diminish their susceptibility to methylation. Additionally, a previously identified pre-30S particle isolated from an \textit{E. coli} culture was assayed for its ability to support methylation by ArmA and found to be inferior to intact 30S particles as a methylation substrate.

Thus, testing of immature particles prepared from \textit{in vitro} and \textit{in vivo} sources suggest that ArmA works very late in the 30S biogenesis pathway. Initiation factor 3 (IF3), a factor that only binds fully mature 30S particles, does not inhibit the ArmA methylation, while kasugamycin methyltransferase (KsgA) abolishes ArmA activity by sharing the same binding site with ArmA. From aforementioned experiments, we conclude that ArmA is most active toward 30S ribosomal subunits that are at or very near full maturation.
Introduction

Ribosome Assembly

Ribosomes are complex and dynamic ribonucleoprotein assemblies which provide the framework for protein biosynthesis in all organisms. Prokaryotic ribosomes consist of two unequal sized subunits; a large 50S subunit and a small 30S subunit. The 'S' refers to Svedberg units of the sedimentation coefficient, which measures how fast cell organelles sediment in an ultracentrifuge. The larger or more spherical the structure, the higher its sedimentation coefficient. Sedimentation coefficients are not additive, so that the 30S and 50S subunits of an assembled ribosome have a sedimentation coefficient of 70S and not 80S. All ribosomes share the same general architecture, with small and large ribosomal subunits composed of rRNA (~65%) and ribosomal proteins (~35%). In *E. coli*, the smaller 30S subunit is composed of the 16S rRNA and 21 proteins, while the larger 50S subunit contains 23S and 5S rRNAs along with 33 proteins\(^1\). Ribosome biogenesis, the process by which ribosomes are synthesized, is very complex and involves transcription of rRNA, pre-rRNA processing, ordered binding of ribosomal proteins and metal ions, modification of both rRNA and ribosomal proteins, and sequential conformational changes\(^1\). Furthermore, all of these events have to be tightly regulated and coordinated to avoid energy losses and imbalances in the cell.
Atomic insights into structural and functional characteristics of ribosomes were gained from the high-resolution crystal structures of individual subunits: the small subunit from the eubacterium *Thermus thermophilus*2,3 and the large subunit from the archaeon *Haloarcula marismortui*4 and eubacterium *Deinococcus radiodurans*5. These structures were followed by that of the 70S ribosome from *T. thermophilus*6 and *E. coli*7. The structure of *T. thermophilus* 70S ribosome in complex with release factors RF1 and RF2 revealed details of interactions of the factors with the ribosome and mRNA8. In addition, Selmer et al. solved the structure of *T. thermophilus* 70S ribosome in complex with tRNA and mRNA9. All of these structures provide insights into the universal mechanism of translation and complex organization of rRNA, ribosomal proteins and ligands. In addition to crystal structures, cryo-electron microscopy studies revealed the flexibility of particles during translation10.

Even though ribosome biogenesis represents one of the fundamental processes in the cell, our understanding of this complex machinery is still somewhat limited. For many decades the structural and functional studies of bacterial ribosomes has been intensively studied. The earliest achievements in ribosome study demonstrated that functional ribosomal subunits could be reconstituted *in vitro* using only the component rRNAs and ribosomal proteins11,12. Following this discovery, 30S subunits were reconstituted using either natural or recombinant proteins13,14 and *in vitro* transcribed 16S rRNA, which lacked modifications15. Particles reconstituted from the recombinant proteins sediment as 30S subunits in a sucrose gradient; however, they are less active in tRNA binding and 50S ribosomal subunit association than naturally purified ribosomes12,14.
Multiple experiments reveal that 30S subunit assembly is highly cooperative and that ribosomal proteins assemble onto the pre-16S rRNA in a cooperative manner, with early binding events organizing the binding sites of late proteins. The small subunit ribosomal proteins have been divided into three groups called primary (1<sup>o</sup>), secondary (2<sup>o</sup>), and tertiary (3<sup>o</sup>) binding proteins. Reconstitution experiments have demonstrated that assembly proceeds with 5′ to 3′ polarity, which coincides with co-transcriptional protein binding, as shown to occur in <i>in vivo</i> ribosomal assembly (Figure 1a). This indicates that the body (5′ region) assembles first, followed by platform (central region), and lastly the head (3′ major region). The final two helices, helix 44 and helix 45, referred to as the 3′ minor region, assemble along the body and platform regions (Figure 1b).

Both <i>in vitro</i> and <i>in vivo</i> studies have tried to analyze the pathway of ribosomal subunit biosynthesis by identifying reconstitution intermediates. Using the <i>in vitro</i> reconstitution system with 16S rRNA and total proteins from 30S subunits (TP30), it was determined that at low temperatures (0°C to 15°C) a particle that sediments as 21S is formed; this particle was termed RI, or Reconstitution Intermediate (Figure 2). The components of RI are the 16S rRNA and ribosomal proteins: S4-S9, S11-S13, and S15-S20. Unimolecular conformational rearrangement in the folding of 16S rRNA yields RI*, which is a more compact particle of identical composition to RI, with a sedimentation coefficient of 26S. The RI to RI* transition is the rate-limiting step in assembly and requires either heat (42°C) or the presence of the DnaK chaperone system. Once this barrier is overcome, remaining ribosomal proteins (S1-S3, S10, S14, and S21) readily assemble, even at low temperatures, leading to formation of 30S subunits. Ribonucleoparticles similar to RI have been observed <i>in vivo</i>, suggesting that the above
described *in vitro* assembly process is valid\textsuperscript{21,22}. There are, however, alternative assembly pathways observed *in vivo*. Bubunenko et al. studied the dependence of the *in vivo* assembly pathway for the 30S platform on the ribosomal protein S15, by a deletion of the gene for S15\textsuperscript{23}. The proteins S6, S11, S18, and S21, which according to the *in vitro* assembly map are dependent on S15 (Figure 1a), were found in normal amounts in purified subunits.

Although the bacterial 30S subunit can be assembled *in vitro* from its rRNA and r-protein components\textsuperscript{11,14}, this process requires nonphysiological conditions, such as high magnesium and monovalent cation concentration\textsuperscript{11}. *In vivo*, however, maturation of rRNA and assembly of the r-proteins into a functional ribosome is a highly complex process\textsuperscript{24} involving multiple accessory proteins\textsuperscript{25}. In the case of the 30S ribosome subunit, a group of proteins is necessary for the proper processing of the pre-16S rRNA. Apart from the nucleases, which are responsible for rRNA cleavage, the *in vivo* assembly requires the presence of the small GTPases Era and RsgA, as well as maturation factors RbfA, RimM, and RimN\textsuperscript{1}. The absence of these proteins results in the accumulation of 17S, a precursor of mature 16S rRNA, which is the product of the RNase III cleavage reaction. While Era and RimN are both essential, RimM, RbfA, and RsgA are not essential, at least under optimal conditions\textsuperscript{26-28}.

In *E. coli*, both tRNA and rRNA (except for the 5S rRNA) are covalently modified during maturation, including both base and ribose modification\textsuperscript{1}. The most common modification in rRNA is base methylation, with few other modifications, including the isomerization of uridine to pseudouridine.
Figure 1. Assembly of the 30S Subunit. (A) In vitro assembly map of the 30S subunit. The primary, secondary and tertiary binding proteins are colored black, red and green, respectively. S6 and S18 bind as a heterodimer\textsuperscript{20}. (B) Domains of the 30S subunit: 5' (blue), central (yellow), 3' major (purple) and 3' minor (red). The structure is adopted from the \textit{E. coli} crystal structure\textsuperscript{7} and modeled with PyMol\textsuperscript{20} (www.pymol.org).
Figure 2. Prokaryotic ribosome assembly. Structures were modeled using PyMol\(^{29}\) (www.pymol.org)
The 16S rRNA contains eleven modified positions, of which ten are base methylations, one is a pseudouridine, and one is methylation on the 2′-O of the ribose (Figure 3). Although these modifications are not essential for protein synthesis in vitro, their localization and conservation suggest that they might be important for folding, assembly, or stability of ribosomes in vivo\(^3^0\). Most of the modified nucleotides are clustered in important functional regions, such as the tRNA binding sites, the decoding region, and the mRNA binding area\(^3^1\) (Figure 4). All the methyl groups in rRNA are added by site-specific methyltransferases, but very limited biochemical data is available regarding their mechanism of action. Some modifications, such as m\(^5\)C967 by RsmB and m\(^7\)G527 by RsmG methyltransferase, are added to the naked 16S rRNA\(^3^2,3^3\). Other modifications are added late during maturation of 30S ribosomal subunits\(^1\). It is possible that the site-specific methyltransferases, which require highly ordered structure and the presence of ribosomal proteins for their activity, recognize a certain structure which is stabilized by the proteins. Only if the correct structure is present, will modifications occur and the assembly process proceed.

**30S Ribosomal Subunit and Its Interaction With Antibiotics**

Antibiotics are natural substances secreted by certain bacteria to protect themselves from other bacteria that are competing for limited nutrients. It has been discovered that antibiotic producer strains have evolved to protect themselves against self intoxication. Later generation of antibiotics used to treat people today are typically derivatives of these natural products. Aminoglycosides have been important antibiotics for treatment of serious bacterial infections, being especially effective against aerobic Gram-negative bacteria\(^3^4\).
Figure 3. Examples of modified nucleotides in 16S rRNA. (A) Uridine. (B) Pseudouridine. (C) Cytidine. (D) N4, 2’-O dimethylcytidine. (E) Guanosine. (F) N7 methylguanosine. Structures were generated using ChemSketch software.
Figure 4. Modified nucleotides during 30S ribosomal subunit maturation. Modified nucleotides are shown as red spheres, 16S rRNA is shown in orange, ribosomal proteins are shown in green, and helix 44 is shown in blue. Decoding region is circled in yellow. The structure is modeled using PyMol\textsuperscript{29} (www.pymol.org)
Since the structures of whole bacterial ribosome subunits and ribosome-antibiotic complexes have been determined, interest in the ribosome as a target for the discovery of the new antibiotics has increased rapidly\textsuperscript{7,35-37}. These findings have determined very precisely the discrete binding sites of several classes of antibiotics inhibiting protein synthesis. Three dimensional structures of ribosome-antibiotic complexes reveal that the interaction of the antibiotic is predominantly with the RNA components of the ribosome.

Aminoglycosides are among the most commonly used broad-spectrum antibiotics to treat aerobic gram-negative infections, such as severe infections of the abdomen and urinary track, bacteremia and endocarditis\textsuperscript{38}. Common to all aminoglycosides is the neamine core, which is composed of a six-membered cyclitol (2-deoxystreptamine) glycosidically linked to a glucopyranosyl. Additional sugars are attached to position 5 or 6 of the 2-deoxystreptamine moiety to form compounds categorized as 4,5- or 4,6-aminoglycosides. Since the amine moieties are mostly protonated under physiological conditions, these polycationic species show a binding affinity for nucleic acids and certain portions of RNAs, especially prokaryotic rRNA. We are studying the 4,6-aminoglycosides, which include but are not limited to, kanamycin, tobramycin, paromomycin, gentamicin and geneticin. These interfere with protein biosynthesis by specifically targeting the ribosomal decoding A site (Figure 5) and inducing codon misreading and amino acid misincorporation. The crystal structure of the highly conserved decoding site in complex with the 4,6-aminoglycoside geneticin (Figure 6) reveals a number of drug-nucleotide contacts, showing the important role of the A site in drug binding\textsuperscript{36,40}. 
Figure 5. Secondary structure of 16S rRNA from *E. coli*. Decoding- A site is shown in red box.
Figure 6. Interaction of geneticin with nucleotides in the A site\textsuperscript{40}. Yellow dashed lines represent hydrogen bonds between geneticin (shown in magenta) and A site residues.
Aminoglycosides target prokaryotic ribosomes over eukaryotic ribosomes, even though the internal loop of A site contains universally conserved nucleotides at six of the seven positions\textsuperscript{41}. The only difference in sequence of the internal loop is at position 1408, which is adenosine in all prokaryotic sequences, but guanosine in all eukaryotic sequences. The A1408-A1493 base pair within the aminoglycoside binding site is critical for high affinity of antibiotics with rRNA\textsuperscript{42,43}. This base pair, along with displacement of two adenosines, A1492 and A1493, toward the minor groove, creates a pocket for aminoglycosides to bind. The aforementioned bases, together with G1494, are important for tRNA binding; hence the binding of aminoglycosides to the A site in the decoding region interferes with the accurate recognition of cognate tRNA by rRNA during translation\textsuperscript{44}.

The structural basis for aminoglycoside mode of action has also been described from the RNA-paromomycin complex, determined by nuclear magnetic resonance spectroscopy, which revealed local conformational changes in the prokaryotic A site of ribosomal RNA upon drug binding\textsuperscript{45}. Binding of paromomycin presumably helps to flip out bases A1492 and A1493 (Figure 7a), which are not well ordered in the structure of the antibiotic-free 30S subunit\textsuperscript{2} (Figure 7b). It is possible that in the absence of the drug some energy is required to flip out A1492 and A1493, so they can contact the tRNA. Most likely, this energy is compensated by the formation of favorable interaction with tRNA. By binding to the decoding site, 4,6-aminoglycosides provoke a structural rearrangement of the decoding site from a state in which it accepts the tRNA to a conformation which is productive for peptide bond synthesis even in the absence of cognate mRNA/tRNA complex.
Figure 7. The decoding A site of the 16S rRNA in the absence of paromomycin\textsuperscript{45} (A) and in the presence of paromomycin (B). The two universally conserved residues A1492 and A1493 are flipped out of helix 44 in the presence of the drug. Paromomycin is shown in magenta (B). Structures were modeled using PyMol\textsuperscript{29} (www.pymol.org).
In the clinical setting bacteria have acquired several resistance mechanisms to 4,6 disubstituted aminoglycosides, including: (a) deactivation of aminoglycosides by N-acetylation, adenyllylation or O-phosphorylation; (b) reduction of the intracellular concentration of aminoglycosides by changes in outer membrane permeability, decreased inner membrane transport, active efflux, and drug trapping; and (c) alteration of the 30S ribosomal subunit target by mutation.

A fourth resistance mechanism is autoprotective in antibiotic producing strains of bacteria. It confers resistance against the toxicity of the bacterium's own secondary metabolites through methylation of their ribosomes by genome-encoded methyltransferases. In the presence of S-adenosyl-L-methionine (SAM), post-transcriptional methylation of specific nucleobases of rRNA in the aminoglycoside binding site of these strains blocks binding of aminoglycoside antibiotics and maintains faithful protein synthesis. Recently, six distinct but related plasmid borne 30S rRNA methyltransferases that confer resistance have been identified in clinical bacterial strains: ArmA, RmtA, RmtB, RmtC, RmtD, and RmtE. All methylate N7 of G1405 and thereby confer resistance to the 4,6-di-substituted aminoglycosides (Figure 8). These six methyltransferases confer no known physiological advantage in the absence of the antibiotics, in contrast to the many endogenous housekeeping methyltransferases that are important for the structure and function of mature rRNA.

It has been shown that the aminoglycoside gentamicin has a high specificity for the A site of translationally active 30S ribosomal subunits. Using footprinting experiments, it has been observed that the 30S subunit was unable to bind gentamicin as a result of G1405 methylation by ArmA. The interaction of 30S subunits with gentamicin was probed by...
Figure 8. Post transcriptional modification of G1405 by Arm/Rmt methyltransferases. The figures were generated using ChemSketch Software.
chemical modification by dimethylsulfate (DMS) and it was found that 30S subunits purified from a strain carrying the armA gene were methylated at G1405. Footprinting of m7G1405 ribosomes did not reveal protection at G1494, even at 100 mM gentamicin, indicating that binding of the antibiotic did not occur. In addition, a mutation in the A site hairpin (G1405C/C1496G) disrupted binding of gentamicin (4,6 disubstituted 2-deoxystreptamine), but did not affect the binding of other classes of aminoglycosides, for example paromomycin, a 4,5 disubstituted 2-deoxystreptamine61.

Arm/Rmt m7G Methyltransferases

A significantly increased spread of N7 G1405 methyltransferases among Gram-negative pathogens has been observed recently. Galimand et al. found the first gene responsible for high-level resistance to 4,6-disubstituted deoxystreptamines and fortimicin and named it aminoglycoside resistance methyltransferase (armA), which encodes a putative 16S rRNA m7G methyltransferase55. Sequence analysis reveals that ArmA belong to the ArmA family of methyltransferases, whose hosts are mainly antibiotic producers Streptomyces and Micromonospora species, as well as pathogens (Figure 9). The overall sequence identity between ArmA and its homologs varies between 21 and 30%, and the levels of similarity are between 37 and 47% 55. Low guanine-plus-cytosine content of armA (30%) distinguishes it from those of antibiotic producing strains, such as grmA (65%) and kgmB (71%), which indicates that armA has not evolved from the resistance gene in the actinomycetes. However, due to the absence of sequences homologous to armA in the data banks, the question remains open as to the identity of the progenitor. The armA gene is borne by conjugative plasmid, in which it is flanked by putative transposable
Figure 9. Sequence alignment of ArmA from *K. pneumonia* (Q7WSN7), RmtA from *P. aeruginosa* (Q8GRA1), RmtB from *E. coli* (Q763K9), RmtC from *P. mirabilis* (Q33DX5), RmtD from *P. aeruginosa* (A0MK31), KgmB from *S. tenebrarius* (Q53316), NbrB from *S. hindustanus* (O52472), GrmA from *M. purpurea* (Q7OKC8), Sgm from *M. zionensis* (Q7MOR2), and FmrO from *M. olivasterospora* (Q2MFZ2). Identical amino acids in all proteins are denoted with a star. The alignment was generated using Clustal X web server.
elements. These elements favor dissemination of *armA*, so it is not very surprising that the gene is found in various members of the *Enterobacteriaceae* family.

The Arm/Rmt family belongs to a well-characterized group of S-Adenosyl-L-Methionine (SAM) dependent methyltransferases, which includes RNA methyltransferases, DNA methyltransferases, protein methyltransferases and small molecule methyltransferases. This group of enzymes contains a Rossman-like fold, which consists of a central β sheet surrounded by a variable number of α helices, and they share several conserved motifs (Figure 10). Many of the Rossman-like methyltransferases have been well characterized structurally and biochemically, and their mechanisms have been explored. In the nucleic acid methyltransferases, the target base is flipped out of a stable conformation in secondary structure in order to be methylated. This mechanism is found in DNA methyltransferases, whose targets reside within the double helix. It is very likely that a similar mechanism is present in the Arm/Rmt methyltransferases, where the N7 position of the modified base G1405 is completely buried within helix 44.

Arm/Rmt methyltransferases recognize assembled 30S ribosomal subunits as their substrates *in vitro*. Despite the high degree of rRNA conservation in the region of methylated G1405, 16S rRNA alone is not an efficient substrate for ArmA. Although 16S rRNA is the target molecule of about eleven enzymes, only in two cases can it serve as an *in vitro* substrate. The housekeeping methyltransferases RsmB and RsmG, which methylate m^5^C967 and m^7^G527 respectively, are enzymes that can utilize 16S rRNA as *in vitro* substrates. More often full or partial assembly is required before full methylation
Figure 10. The crystal structure of truncated ArmA$^{65}$ (wheat) superimposed on RmtB$^{65}$ (magenta) and Sgm$^{66}$ (green). Figures were generated using PyMol$^{29}$ (www.pymol.org).
can occur, suggesting that rRNA modification is integrated into ribosome assembly *in vivo* by being able to recognize more complex substrates. In some cases, 30S ribosomal subunit in the presence of high Mg\(^{2+}\) concentration fails to serve as an efficient substrate, possibly indicating that a low Mg\(^{2+}\) concentration induced or translationally inactive conformation is recognized by the modification enzyme, but the high Mg\(^{2+}\) or translationally active conformation is not. Biochemical studies indicate that these two states are structurally very similar, except in the decoding region.

This dynamic decoding region corresponds to the region of 30S ribosomal subunits that has the highest density of modified nucleotides (Figure 4). For that reason, it is not very surprising that there is variable access to nucleotides that are to be modified. For example, the housekeeping methyltransferase KsgA, which modifies nucleotides A1518 and A1519, requires relaxation of tertiary interactions between helix 44 and helix 45 to methylate the bases. Likewise, some conformational accommodations on the part of 16S rRNA are presumably required for Arm/Rmt to methylate its target nucleotide G1405. The structure of fully formed translationally active 30S ribosomal subunit reveals that G1405, the target nucleotide for Arm/Rmt methyltransferases, is buried as a consequence of direct tertiary interaction with nucleotide A1518 of the helix 45 (Figure 11). The sequestered position of the Arm/Rmt target nucleotide G1405 and the heavy traffic of modifying enzymes converging on this region, suggest that conformational rearrangement of 16S rRNA might be required for these enzymes to access their target nucleotide. Liou et al. showed that isolated helix 44, where G1405 lies, is not a possible substrate for ArmA. This indicates that Arm/Rmt methyltransferases, as well as modification reactions of 16S rRNA during 30S ribosome assembly, are sensitive to a number of solution variables and...
Figure 11. Tertiary interaction between G1405 (red sphere) and A1518 (blue sphere). Both nucleotides are circled in magenta. Black arrow points to the modified position N7 of G1405. Insert shows the hydrogen bonds shared between the same nucleotides. Figures were generated using PyMol\textsuperscript{29} (www.pymol.org)
assembly states. It is very likely that efficient substrates in many ribosomal modification reactions are often some assembly state other than 16S rRNA or 30S ribosomal subunit.

**Unanswered questions**

Aminoglycosides are used extensively for the treatment of severe infections caused by Gram-negative bacteria. Recently, however, certain bacterial pathogens have developed resistance after acquiring genes for methyltransferases that catalyze post-transcriptional methylation of N7-G1405 in the 30S ribosomal subunit. Inactivation of this enzymatic activity presents an important challenge and opportunity to develop effective inhibitors that will retain the effectiveness of aminoglycoside antibiotics. For that reason, we were interested in biochemically and structurally characterizing this group of enzymes in order to better understand their mechanism of action. Recent studies on ArmA homologs have opened up intriguing lines of inquiry. However, there is a lack of information about the exact mechanism of Arm/Rmt-catalyzed reactions, which is not surprising given the short time that this group of enzymes has been studied. One unresolved question is the order of substrate binding: Is 30S ribosomal subunit or SAM required to bind first and create the binding site for the second substrate?

In addition, the exact nature of Arm/Rmt’s complex substrate requirements is not fully understood. The enzyme cannot methylate the isolated helix 44, 16S rRNA, 50S ribosomal subunit or 70S ribosome, but the fully formed 30S ribosomal subunit is methylated *in vitro*. This indicates that Arm/Rmt requires a highly structured ribonucleoprotein particle as its optimal substrate, where access is blocked by the 50S ribosomal subunit. The window during ribosome biogenesis between free RNA and mature
30S subunit when Arm/Rmt can access nucleotide G1405 and transfer the methyl group remains to be defined. It was shown that the late acting ribosomal methyltransferase RsmF, which methylates \textsuperscript{5}C1407 during ribosome biogenesis, is obstructed by the presence of Sgm, an ArmA homolog\textsuperscript{73,74}. In contrast, the two methylations at 16S rRNA nucleotide \textsuperscript{4}Cm1402 are unaffected by the presence of Sgm\textsuperscript{73,75}. This indicates that Arm/Rmt acts later during ribosome biogenesis and that interaction between housekeeping methyltransferases and resistance methyltransferases plays an important role in Arm/Rmt methyltransferases mode of action.

**Scope and objectives**

Housekeeping methyltransferases play a role in prokaryotic ribosome maturation and are synchronized with rRNA processing, ordered binding of ribosomal proteins and conformational changes\textsuperscript{31,74,76}. In 16S rRNA, many of the base modifications are clustered in the phylogenetically conserved and functionally essential decoding region of the 30S subunit\textsuperscript{77}. The aminoglycoside binding pocket and G1405, the target of ArmA, are located within the A-site of the decoding region and are flanked by several methylated nucleotides: C1402, C1407, U1498, A1518, and A1519. This tight clustering very likely imposes an evolved order of methylation steps to avoid methyltransferase crowding and possible obstructive stereoelectronic effects to downstream methylations introduced by the modifications. Such constraints would require synchronization of ArmA with the other housekeeping methyltransferases. The sequestered position of the Arm/Rmt target nucleotide G1405 in the crystal structure of the 30S subunit and the heavy traffic of
modifying enzymes converging on this region, suggest that conformational rearrangement of 16S rRNA might also be required for these enzymes to access their target nucleotide.

Given that very limited biochemical data has been reported on the function of this group of enzymes, this study is intended to further investigate the Arm/Rmt mechanism of action. The substrate specificity and requirements of ArmA were investigated to both characterize the enzyme and to resolve the conundrum of how they interact with their target nucleobase. We determined the Mg\(^{2+}\) dependence of ArmA activity and the effects of gentamycin, kasugamycin resistance methyltransferase (KsgA) and initiation factor 3 (IF3) binding to 30S subunits on ArmA activity. We also tested LiCl treated 30S particles and in vivo derived pre-30S ribosomal subunits as ArmA methylation substrates. We find that very late steps in 30S ribosomal maturation are prerequisites for these methyltransferases to carry out their modification reaction, since the best substrate for ArmA is a highly structured ribonucleoprotein particle, very close in conformation to the fully mature 30S substrate. Conformational changes, either very late in the pathway of 30S ribosomal subunit assembly or off-pathway, appear to be necessary for Arm/Rmt methyltransferase to access the completely buried G1405.
Cloning, Production, and Initial Characterization of Arm/Rmt Enzymes

For proteins to be analyzed using informative structural methods such as X-ray crystallography or NMR, they have to be highly soluble and stable over a reasonable period of time. While sometimes proteins of interest behave in this way, more often protein purification can be challenging and difficult. Preparation and expression of proteins can have many obstacles, such as protein aggregation and proteolytic degradation. Protein insolubility at higher concentration can present problems, especially if the aim of the study is to structurally characterize the protein. In order to improve the yield of soluble protein, many strategies are used, including the optimization of protocols for expression and purification of proteins, or expressing the desired proteins in alternative host cells.

The purification and expression of the ribosomal methyltransferase, RmtA, was initially undertaken for purposes of structural characterization of this group of enzymes. *Pseudomonas aeruginosa* pJ31:6303- *rmtA* was synthesized chemically (DNA. 2.0 Inc.) to incorporate NdeI and XhoI cleavage sites for cloning. These enzymes (New England Biolabs) were used for digestion of the synthetic DNA and the resultant fragments were ligated into the plasmid vector pET15b, which had been digested with these same enzymes (NdeI and XhoI). *Escherichia coli* XL1-Blue (Stratagene) was transformed with the ligation mixture and transformants were selected on Luria-Bertani agar plates supplemented with 30 mg/ml of kanamycin.
The pET15b-\textit{rmtA} plasmid containing an N-terminal His-tag coding region was isolated using a Qiagen Mini-prep kit. The correct ArmA sequence was confirmed by the Nucleic Acid Research Core Facility at Virginia Commonwealth University. The plasmid was transformed into both HMS17 and BL-21(DE3) cells for protein production, but only the latter produced colonies. This construct gave very low RmtA expression, so other plasmid constructs made by cloning \textit{rmtA} into pET20-b, pET21-c and pET31-b, were tested for better RmtA expression. Unfortunately, these three constructs showed even lower expression compared to the pET15-b- \textit{rmtA}, with most of the enzyme being in inclusion bodies. Even when the enzyme was produced under conditions of lower temperatures at 18\textdegree C/ 25\textdegree C or lower concentration of IPTG (0.1 mM), the solubility of RmtA did not change significantly. In the end, the original pET15b N-terminal His-tag construct yielded the most soluble protein of the three constructs, but the enzyme remained too unstable at higher concentrations (>5 mg/ml) to be amenable to structural characterization. Even the use of multiple solubility screens, such as different pH and salt concentrations, failed to increase protein solubility enough to screen for crystallization.

\textit{Escherichia coli} DH10B+ pJ210:10629- \textit{rmtC} was also obtained from DNA 2.0 Inc. NdeI and XhoI (New England Biolabs) were used for digestion of the synthetic DNA and the digest ligated into the plasmid vector pET29-b, which carries a C-terminal His tag coding region. \textit{E. coli} XL1-Blue (Stratagene) was transformed with the ligation mixture, and transformants were selected on Luria-Bertani agar plates supplemented with 100 mg/ml of ampicillin and 30 mg/ml chloramphenicol. The sequence of isolated pET29b- \textit{rmtC} plasmid was confirmed by the Nucleic Acid Research Facilities at Virginia Commonwealth University. However, RmtC engineered with a C-terminal His-tag showed
low *in vitro* methylation activity. This may be due to the His-tag affecting the correct folding of the protein or the binding of the two substrates, 30S ribosomal subunit and S-Adenosyl-L-Methionine (SAM). Therefore, the *rmtC* gene was ligated into pET15b, which contains an N-terminal His-tag coding region. The correct *rmtC* sequence was confirmed by Nucleic Acid Research Facilities at Virginia Commonwealth University. RmtC was over-expressed with 1 mM IPTG and purified by Ni-NTA affinity chromatography. Protein purification attempts were made more difficult by insolubility of the overexpressed RmtC, so purification conditions were optimized for production of soluble protein. Under different buffer conditions, RmtC was not stable in the absence of at least 10% glycerol and reducing agent, either dithiothreitol (DTT) or β-mercaptoethanol (BME). Even under these conditions, the protein precipitated out when stored for more than one week at 4°C.

*Acinetobacter baumannii* pUCarmA1-*armA* construct was obtained from Dr. Yohei Doi, of the University of Pittsburgh School of Medicine. The gene encoding *armA* was inserted into pET15b as an NdeI-XhoI fragment with an N-terminal His tag. The correct ArmA sequence was confirmed by Nucleic Acid Research Core Facility at Virginia Commonwealth University. The plasmid was transformed into BL-21(DE3) cells for protein production. ArmA was overexpressed with 1 mM IPTG, purified by Ni-NTA affinity chromatography eluting with 0.3M Imidazole. Protein was estimated to be >95% pure using SDS-PAGE analysis and stable for at least a few weeks at 4°C. Since ArmA with an N-terminal His-tag showed improved stability relative to the Rmt constructs over a longer period of time, we decided to conduct our future experiments using this enzyme. *In vitro* activity assays confirmed that the correct enzyme was purified (see below).
**In vitro Analysis**

The relative enzymatic efficiency of RmtA, RmtC and ArmA in methylation of *E. coli* wild type 30S ribosomal subunits in an *in vitro* activity assay was determined. Substrate 30S subunits were prepared from wild type *E. coli* as described in the experimental section. Incorporation of $^3$H-methyl from labeled SAM by each enzyme was measured after an incubation of one hour. Negative control experiments were performed with 30S subunits in the absence of enzymes. Also, the same assay was attempted in the absence of 30S ribosomal subunits, but in the presence of methyltransferases. All experiments were performed with 10 pmol of 30S substrate and 10 pmol of enzyme. *In vitro* activity assays for RmtA and RmtC showed somewhat lower final incorporation of $^3$H- SAM compared to ArmA (Figure 12). This might be due to the solubility problems seen for RmtA and RmtC. It is likely that the enzymes started forming aggregates during the *in vitro* assay (as was also noticed during the purification step) leaving only a fraction of soluble RmtA/RmtC available to methylate the 30S ribosomal subunit. Another reason for differences in incorporation of $^3$H-methyl from labeled SAM could be the use of different 30S ribosomal subunit preparations. Nevertheless, it seems that ArmA is simply more efficient than the other two enzymes.

The Arm/Rmt enzymes transfer only one methyl group from the SAM molecule to guanosine 1405. The exact mechanism of transfer has not yet been established. Question remains as to a preferred order, if any, of substrate binding. So, our next question was to see if the order of substrate addition for the 30S ribosomal subunit and SAM would influence the incorporation of $^3$H from the labeled SAM by ArmA (Figure 13 A). All experiments were performed with 10 pmol of 30S substrate and 10 pmol of enzyme, and
the data was fit using logarithmic curve. With stoichiometric amounts of protein relative to 30S, the time-course of methylation was essentially indistinguishable among the different experiments, confirming that the order of substrate addition did not influence the final level of methylation. In addition, the rate of incorporation of methyl group does not change when substrates are added in a different order (Figure 13B). This result is, however, open to interpretation. Due to the complex mechanism, it is possible that the addition of the second substrate, but not the first one, represents the slow step in the reaction. In our experiment, we were limited in detecting only the net chemical reaction, so additional methods might be necessary to determine which step in the reaction is rate-limiting.

**Mg\(^{2+}\) Dependence of ArmA Activity**

It is known that the conformational state of the 30S ribosomal subunit is sensitive to the concentration of Mg\(^{2+}\) and NH\(_4\)Cl\(^{79,80}\). High Mg\(^{2+}\) concentrations stabilize an active state of 30S, while low concentrations of Mg\(^{2+}\) stabilize a translationally inactive conformation of the 30S subunits \(^{69}\). It has been determined that ArmA is active in methylating 30S ribosomal subunits at higher concentration of Mg\(^{2+}\)\(^{61}\). In order to further explore the magnesium dependence of ArmA activity, we varied the concentration of this divalent cation in our assays (Figure 14A). The broad plateau of maximal ArmA activity in the range of 10 - 15 mM Mg\(^{2+}\) indicates that the enzyme methylates the translationally active form of the 30S subunit. However, ArmA retains 1/2 of its maximal activity at 4 mM Mg\(^{2+}\), where the translationally inactive form of the 30S subunit predominates, suggesting that ArmA recognizes both the translationally active and inactive forms.
Figure 12. *In vitro* methylation of 30S by RmtA (A), RmtC (B), and ArmA (C). All reactions were performed for 1 hour at 37 °C in Buffer ‘R’ and contained $^3$H-SAM.
Figure 13. *In vitro* methylation of 30S subunit. Time-course assays for ArmA (A) measured over 90 minutes. Blue diamond indicates assays containing 10 pmol 30S added first, then 10 pmol enzyme, and SAM; red square indicates assays containing 10 pmol of enzyme added first, then SAM and 10 pmols 30S; green triangle indicates assays containing 10 pmol 30S added first, then SAM and 10 pmols of enzyme; purple cross indicates assays containing 10 pmol of enzyme added first, then 10 pmol 30S and SAM. Blue star indicates assays containing SAM added first, then 10 pmol 30S and 10 pmol of enzyme; orange circle indicates assays containing SAM added first, then 10 pmol of enzyme and 10 pmol 30S. (B) *In vitro* methylation assay measured in the first 10 minutes. The data was fit using logarithmic (A) or linear (B) curve. The same legends applied as in the part (A).
As a further test of whether ArmA recognizes the low Mg\textsuperscript{2+} translationally inactive form of the 30S subunit, we utilized kasugamycin resistant (ksg\textsuperscript{R}) 30S subunits, which lack methyl groups on A1518 and A1519. On the basis of the known tertiary interaction involving modified nucleotides A1518/A1519 with helix 44, these methylations are expected to stabilize the active conformation of the 30S subunits and their absence should increase the proportion of translationally inactive form in the low Mg\textsuperscript{2+} concentration transition range\textsuperscript{2,7}. The Mg\textsuperscript{2+} dependence of ArmA activity is similar for the wild type and ksg\textsuperscript{R} forms of 30S subunit at high Mg\textsuperscript{2+} concentrations: both have broad maxima centered around 8 – 10 mM. However, the activity profile for the ksg\textsuperscript{R} substrate (Fig. 14B) is skewed to significantly higher activity at lower Mg\textsuperscript{2+} concentrations (4 - 6mM) than the wild type. While the Mg\textsuperscript{2+}-dependence of ArmA activity is somewhat sensitive to the presence of A1518 and A1519 methylation, it is clear that ArmA is capable of recognizing both the inactive and active forms of 30S subunits. Mathew Baker from our lab also tested the ArmA ortholog RmtA for activity in the presence of low and high Mg\textsuperscript{2+} concentrations and obtained similar results (data not shown).

**Conclusions**

Arm/Rmt enzymes identified in clinical strains post-transcriptionally methylate nucleotide G1405 at the N7 position in the presence of SAM, and confer resistance to the 4,6-disubstituted aminoglycosides\textsuperscript{61,81}. These methyltransferase enzymes provide no known physiological advantage in the absence of the antibiotics and in that sense they differ from many endogenous, housekeeping methyltransferases, which are important for both the structure and function of the rRNA\textsuperscript{1}. 


Figure 14. Effect of Mg$^{2+}$ on Arm activity. *In vitro* methylation of wild-type 30S (A) and ksg$^R$ 30S (B)
The decoding region of *E. coli* 16S rRNA alone, where the modified G1405 lies, contains several nucleotides that are methylated by endogenous methyltransferases, such as C1402, C1407, and U1498. With all of these bulky methyltransferases operating in this small region of 16S rRNA, it is difficult to picture another enzyme finding its way to modify G1405. Substrate recognition by the Arm/Rmt methyltransferases might be complex. Arm/Rmt is able to methylate 30S ribosomal subunit *in vitro*, but it does not methylate either 70S ribosome or 16S rRNA. In addition, isolated helix 44 is not an efficient substrate for ArmA, proving that the enzyme recognizes structured ribonucleoprotein *in vitro*. The reported k$_{cat}$ value for ArmA, 0.2 min$^{-1}$ is comparable to turnover numbers for RsmE, 0.042 min$^{-1}$ and RrmJ, 0.064 min$^{-1}$, indicating that ArmA, like RsmE and RrmJ, might require highly assembled ribosomal particles for methylation.

In contrast to ArmA, RmsB and RlmD methyltransferases have significantly higher turnovers *in vitro*: 2.6 min$^{-1}$ and 3.6 min$^{-1}$, respectively. However, this is somewhat expected, since these latter enzymes act on free rRNA and may not require highly structured substrates. If we take into account that the time required for 30S subunit maturation *in vivo* has been reported to be only a few minutes, ArmA does not act very slowly *in vivo*. It is clear from the 30S crystal structure that G1405, the target nucleotide for Arm/Rmt methyltransferases, is buried as a consequence of direct tertiary interactions with nucleotides A1518 and A1519 of helix 45. It has not been confirmed if there is an obligate order to substrate binding to allow ArmA to access the buried nucleotide. Enzyme binding to 30S subunits most likely requires either conformational rearrangement of 16S rRNA or the substrate that we use for an *in vitro* study is different in some way from the one that is modified during ribosome assembly *in vivo*. Our results demonstrate that ArmA
is able to methylate both active and inactive conformations of 30S ribosomal subunits in vitro, and that the order of substrate addition does not influence the final level of methylation. However, the complexity of the Arm/Rmt methyltransferases imposes the need to further explore and understand their mechanism of action.

**Experimental**

**Cloning**

A synthetic rmtA gene was obtained from DNA 2.0. The 776 base-pair rmtA gene was amplified by PCR using the following primers, purchased from Integrated DNA Technologies: 5’-CTG ACT GCA ATA TGA GCT TTG ACG ATG CCC-3’ and 5’- CTG CCT CGA GTC ACT TAT TCC TTT TTA TCA TGT ACA C-3’. The gene was cloned into the pET15b vector (Novagen) as an NdeI/XhoI fragment for expression as an N terminal His tagged fusion construct. The recombinant plasmid was sequenced (Nucleic Acids Research Facilities, VCU) to confirm the presence and correct sequence of the insert.

A synthetic rmtC gene was obtained from DNA 2.0. The 879 base-pair rmtC gene was amplified by PCR using the following primers, purchased from Integrated DNA Technologies: 5’- ATA CAT ATG AAA ACC AAC GAT AAT TAT ATC GAA -3’ and 5’- ATA CTC GAG TCA CAA TCT CGA TAC GAT AAA ATA CAT-3’. The gene was cloned into pET15b vector (Novagen) as an NdeI/XhoI fragment for expression as a His tagged fusion construct. The correct clone was confirmed by sequencing (Nucleic Acids Research Facilities, Virginia Commonwealth University).

*Acinetobacter baumannii* pUCarmA1-ArmA construct was obtained from Dr. Yohei Doi, from the University of Pittsburgh School of Medicine and was confirmed by
sequencing. The gene encoding armA was inserted into pET15b as an NdeI-XhoI fragment. The isolated pET15b-armA plasmid contained N-terminal His-tag coding region. The correct ArmA sequence was confirmed by the Nucleic Acid Research Facilities at Virginia Commonwealth University.

**Protein expression and purification**

pET15b-RmtA and pET15b-RmtC plasmids were transformed into BL-21 (DE3) Rosetta II cells for overexpression. Cell cultures were grown to an OD600 of 0.6 in the presence of ampicillin and chloramphenicol and induced with 1 mM IPTG (Sigma-Aldrich). After 4 hours at 37°C, cells were harvested by centrifugation. Pellets were resuspended in lysis buffer (50 mM NaPO4, 500 mM NaCl, 10 mM imidazole, 10 % glycerol, 1 mM DTE pH 7.5), broken with two passages through an Emulsiflex cell breaker (Avestin), and centrifuged to remove cell debris. Cleared lysate was loaded onto a HiTrap Chelating column (Amersham) equilibrated with 0.1M NiSO4, washed with lysis buffer and the proteins were eluted with elution buffer (50 mM NaPO4, 500 mM NaCl, 300 mM imidazole, 10% glycerol, 1 mM DTE pH 8.0).

The pET15b-ArmA plasmid was expressed in BL21 (DE3) cells (Stratagene). The cells were grown at 37°C to an OD600 of 0.6 in the presence of ampicillin. The protein was induced with 1 mM IPTG and transferred to 37°C for 4 hours. Cells were harvested and broken as for RmtA and RmtC. Purification was carried out by affinity chromatography using a Ni-NTA chromatography column; buffers were the same as above. Proteins were estimated to be >95% pure by SDS-PAGE analysis. Protein concentration was measured using the absorbance at 280 nm and the extinction coefficient, using protein calculator v3.3, for each protein. ([http://www.scripps.edu](http://www.scripps.edu))
30S purification

For in vitro activity assay, 30S ribosomal subunits from the MRE600 strain of E. coli were prepared as described. For 30S subunits lacking methylation at A1518 and A1519, an MRE600 strain resistant to the antibiotic kasugamycin were used. Subunits from this strain are referred as 30S-ksgR.

Wild-type purified subunits were dialyzed into reaction buffer (40 mM Tris, pH 7.4; 40 mM NH4Cl; 8 mM MgOAc; 1 mM DTE) and stored at −80°C in single-use aliquots. The ksgR purified subunits were dialyzed into reaction buffer (40 mM Tris, pH 7.4; 40 mM NH4Cl; 4 mM MgOAc; 1 mM DTE) and stored at −80°C in single-use aliquots. 30S concentration was estimated by measuring the absorbance at 260 nm and using a relationship of 67 pmol 30S per 1 unit of optical density.

In vitro analysis

The in vitro assay was adapted from Poldermans et al. (1979). Time-course reactions were performed in 50 µl volumes containing 40 mM Tris, pH 7.2, 40 mM NH4Cl, 8 mM MgOAc, 1 mM DTE, 0.02 mM 3H-methyl-SAM (780 cpm/pmol; MP Biomedicals), 10 pmol 30S subunits, and 10 pmol enzyme. Buffer and reagents were pre-warmed to 37°C and added into pre-warmed tubes to minimize any lag in the reaction start. At each of the designated time points 10 µl of 100 mM unlabeled SAM (Sigma- Aldrich) was added to tubes to quench the reaction. The quenched reactions were deposited onto DE81 filter paper (Whatman), washed twice with ice-cold 5% TCA, and rinsed briefly with ethanol. Filters were air-dried for one hour, placed into scintillation fluid, and counted.
ArmA Interaction With its Substrates - 30S subunit and S-Adenosyl-L-Methionine

Introduction

The interaction between ArmA and 30S subunit is complex and details of the mechanism of ArmA action are not known, in part because of the limited biochemical data on this group of enzymes. Neither 16S rRNA alone nor 70S ribosome is a substrate for Arm/Rmt methyltransferases, while assembled 30S subunit is methylated by purified ArmA in vitro. If we examine the crystal structure of fully formed translationally active 30S subunits, we notice that the target nucleotide G1405 is sequestered from the surface and is inaccessible for modification by ArmA (Figure 15). However, Agarwal et al, using cryo-electron microscopy revealed that the 30S subunit is conformationally flexible, with the conformational changes mainly localized in the neck, the platform and shoulder regions. Since the ArmA target nucleotide lies in helix 44, a highly flexible region of 30S, the enzyme presumably induces a conformational change required for the methylation of G1405. It is also possible that the mechanism of ArmA action includes the flipping out of the modified base G1405. Base flipping has been documented for both DNA- and RNA-modifying enzymes and is believed to be the dominant mechanism used by these enzymes to access target bases that are normally inaccessible due to secondary and/or tertiary interactions in the nucleic-acid molecule.
Figure 15. Modified nucleotide G1405 (circled in red) in the 30S ribosomal subunit. The 16SrRNA is shown in orange and the ribosomal proteins are shown in grey. The red arrow points toward the buried N7 position of G1405. Structures were generated using PyMol\textsuperscript{29}. 
To gain a better understanding of the complex mode of ArmA action, we undertook investigation of the ArmA interaction with the 30S ribosomal subunit. Directed hydroxyl radical probing, followed by primer extension analysis, was used to investigate ArmA interaction with its substrate 30S ribosomal subunit. Directed hydroxyl radical probing generates low resolution (10–50 Å) information about the part of a nucleic acid that is in the vicinity of a defined site on a protein or other nucleic acid. This approach has been used to study the 16S rRNA environment around individual ribosomal proteins in the 30S subunit, elongation factors, initiation factors, release factors, ribosome recycle factors, and ribosomal methyltransferases. This approach was used to explore the sites of interaction between ArmA and 30S subunit to determine the specific contact regions between the two.

**Results**

**Directed Hydroxyl Radical Probing**

To probe 16S rRNA with hydroxyl radicals, Fe(II) is tethered to specific positions on the surface of a protein by reaction of the Fe(II)-loaded linker, 1-(p-bromoacetamidobenzyl)-EDTA with unique cysteine residues. To produce ArmA with different unique single cysteine residues, the only naturally found cysteine in ArmA at position C115 was mutated to alanine. This mutant with no cysteine (cys-less) was used as our control sample and served as background when comparing the other cleavage sites on the primer extension gel. The cys-less mutant was used to construct double mutants and to insert single cysteine residues at positions 43, 59, 64, 190, 220, and 234 (Y43C, Y59C, L65C, V190C, M220, G234C). These positions on ArmA are shown in Figure 16.
Figure 16. ArmA cysteine mutants. Backbone atoms of residues which were mutated to cysteine are shown as spheres. Y59C is yellow, L65C is orange, C115 (wild type) is cyan, V190C is blue, and G234C is magenta. Y43C is not shown in the figure since the N terminal truncated form of ArmA58 was used. The structure was generated using PyMol\textsuperscript{29}. 
We chose these specific residues based on several criteria. First, we wanted to mutate residues that were not conserved in ArmA, thus limiting the likelihood that they would interfere with protein folding and function. Second, we wanted to mutate residues that are surface exposed and readily accessible to solvent. Lastly, we chose residues that were widely spaced throughout ArmA, so that the information we gained from the hydroxyl radical experiment would not be redundant. Before starting the experiment, we checked in vitro methylation activity of each mutant and found that all except one, ArmA C115A M220C, could fully methylate 30S subunit (Figure 17). We discontinued working with this ArmA double mutant, since it precipitated out during purification steps. The six selected mutants were purified as previously described in Chapter 2.

In this technique, hydroxyl radicals are generated locally from Fe(II) tethered to a single position in ArmA, resulting in cleavage of the rRNA backbone at positions proximal to the Fe(II) ion. Because of the short lifetime of hydroxyl radicals in aqueous solution, cleavage is usually restricted to positions in the RNA that are within about 10 Å of the Fe(II) ion\textsuperscript{88}. Fe(II) is tethered to the unique cysteine residue at specific sites in ArmA via 1(p-bromoacetamidobenzyl)-EDTA (BABE), a reagent that has been successfully used to map intramolecular distances in proteins\textsuperscript{98}. Iron-derivatized ArmA is incubated with a fully formed 30S subunit to create a complex (Figure 18). Once the complex between ArmA and 30S subunit is formed, hydroxyl radicals are generated by the Fenton reaction in the presence of H\textsubscript{2}O\textsubscript{2} and ascorbic acid\textsuperscript{99}. After cleavage the RNA is extracted from the 30S subunits, annealed with labeled primers, and analyzed by primer extension, an extremely sensitive technique for detecting breaks in the RNA backbone (Figure 19).
Figure 17. *In vitro* activity assay of ArmA cysteine mutants.
Figure 18. Site directed hydroxyl radical probing. Single cysteine residues (shown as yellow spheres) are mapped onto the ArmA structure (A). Fe(II) BABE is attached to each single cysteine residue, represented as a black star (B). ArmA single cysteine residues are incubated with 30S subunit to form a complex (C). In the presence of H₂O₂ and Ascorbic acid, hydroxyl radicals are generated to cleave 16S rRNA backbone (D).
Figure 19. Primer extension. Cleaved 16S rRNA is first annealed with radio labeled primers and then extended using reverse transcriptase in the presence of free nucleotides. The product is analyzed on sequencing gel as shown in Figure 20.
In this work, synthetic DNA oligomers were used to anneal to target RNA template sequence and prime DNA polymerization by reverse transcriptase. These primers were generally 17 nucleotides long and were complementary to a single region of the target 16S RNA. Cleavages of the RNA were indicated by the appearance of additional bands, above background, in the autoradiogram, which represent abortive cDNA transcripts ending at the position of RNA template cleavage. Portions of the RNA backbone which are in proximity to the Fe(II) will be cleaved more often than positions which are further away. Therefore, more cleavage events occurring at a particular site correspond to a heavier band on the gel. Figure 20 shows representative autoradiograms, which include two dideoxy sequencing lanes, a control cys-less ArmA lane and experimental lanes from the ArmA-Fe(II) mutants. Autoradiography of the gel with Kodak Hyperfilm was typically carried out overnight at room temperature. However, the length of time required for appropriate exposure varied for different primers. Cys-less ArmA is considered to be the background for the other lanes, since the bands appearing in this lane do not correspond to direct probing. Band intensities are correlated with the distance between the RNA target and the position of the Fe(II) on ArmA. When probing was initiated from Fe(II)-modified ArmA proteins (Figure 16) bound to the active 30S subunit, both unique and in some instances overlapping, cleavage patterns were obtained from three of the six Fe(II)-derivatized ArmA proteins.

**RNA Footprinting Analysis of ArmA Interaction With the 30S Subunit**

To identify the site of interaction between ArmA and the 30S subunit, hydroxyl radical probing initiated from Fe(II)-modified ArmA proteins bound to the *E. coli* 30S subunits was performed as described above. For this experiment, different primers were
used to scan the whole 16S rRNA, but only primers 480, 939, and 1257 showed reproducible cleavages (Figure 20). The cleavage patterns obtained from different Fe(II) - derivatized ArmA proteins show little similarity and are somewhat dispersed throughout the 16S rRNA with no evidence of clustering at a specific site on 30S subunit. Cleavages were seen in helices 25 and 34, in the loops of helices 14 and 15, and in the base of helices 24, 25 and 27 (Figure 21). In addition, we observed cleavage sites along helix 44, but the helix 44 results were not reproducible when the probing was repeated, so the data were excluded.

When the cleavage data were mapped onto the structure of the three – dimensional fully mature 30S subunit, we noticed multiple binding sites dispersed around the modified nucleotide G1405 (Figure 22A). Some of the cleavages, such as the ones seen in helices 24 and 27, were more than 30 Å away from the modified nucleotide G1405. In addition to these distal sites, cleavages were detected on the opposite face of the 30S ribosomal subunit relative to the target nucleotide, close to the binding site of the ribosomal protein S15 (Figure 22B). However, the most surprising cleavages identified from this experiment were completely buried inside helix 34 and inaccessible to ArmA in the translationally active 30S conformation. In addition, we observed cleavage sites along the helix 44 (data not shown); however, the helix 44 results were not reproducible when the probing was repeated. It should be noted that these experiments were performed in the absence of S-Adenosyl Homocysteine (SAH) as a surrogate second substrate (inhibitor). However, the presence of this second ‘substrate’ is unlikely to be critical for ArmA binding to the 30S subunit, since similar ambiguous results were obtained for Sgm footprinting experiments,
Figure 20. Primer extension analysis of directed hydroxyl radical cleavage of 16S rRNA in the translationally active 30S subunits from Fe(II)-ArmA. Cleavage sites and the sequence numbers of nucleotides within 16S rRNA are denoted by bars on left and right of figures, respectively. A and G are sequencing lanes; Fe(II)-ArmA–30S complexes are: Cys-less, a Fe(II)-BABE treated cysteine-free mutant of ArmA; Fe(II)-Cys43-ArmA; Fe(II)-Cys59-ArmA; Fe(II)-Cys65-ArmA; Fe(II)-Cys115-ArmA; Fe(II)-Cys190-ArmA; Fe(II)-Cys234-ArmA. Primers are 480 (A), 939 (B and C) and 1257 (D).
Figure 21. Directed hydroxyl radical cleavage sites from Fe(II)-ArmA (shown in boxes) on the secondary structure of 16S rRNA
Figure 22. Directed hydroxyl radical probing mapped on the 30S subunit from ArmA. The cleavage sites are shown on the three-dimensional structure of E.coli 30S structure⁷ (A) and include 180° rotation view of the same figure (B). The modified nucleotide G1405 is circled in red.
which were done in the presence of 1 mM SAH. The multiple and dispersed binding sites seen in the Sgm-30S complex, observed at helices 24, 42, 43, and 44, indicate that SAH does not influence binding of ArmA (or Sgm) to the 30S subunit.

This ambiguous pattern of ArmA interaction with the 30S subunit could be due to binding at multiple non-specific sites and/or to large conformational changes in the 30S subunit upon ArmA binding. ArmA is a monomer under the conditions used for these experiments, which excludes the possibility of extended oligomers modifying at sites remote from the methylation site. The data obtained from this experiment correlate well with the results reported by Husain et al., where chemical footprinting with CMCT (specific for nucleotides G and U) and DEPC (specific for nucleotide A) were carried out on E. coli 30S subunits to determine the sites of interaction between 30S and the sisomicin-gentamicin methyltransferase Sgm, an ArmA homolog. In contrast to the ArmA – 30S binding sites, Sgm cleavages extended to helix 44, where the modified nucleotide G1405 lies. They reported that conformational changes occur in the 30S subunit, upon its interaction with Sgm, that allow the enzyme to access the target nucleotide G1405, which is otherwise buried in the crystal structure of 30S subunit.

To gain insight into possible ArmA and target - base interactions, we tried to build a model using the program PyMol. However, we were not able to position G1405 into the active site of the ArmA – SAM complex due to multiple close contacts. First, we tried to build the ArmA -30S model using the crystal structure of fully formed translationally active 30S ribosomal subunit. In this 30S structure, the modified base was completely sequestered in the helix 44 and was not accessible to ArmA for transfer of the methyl group. Second, our directed hydroxyl radical data revealed that ArmA reached mostly the
solvent – exposed face of helices. This implied that the target base was never closer than 20Å to the methyl group of SAM. Third, even if we minimized the distance between the methyl group of SAM and the target nucleotide, the base would need to be rotated out of helix 44 and N7 position of G1405 exposed to the surface. This base flipping mechanism would be similar to the ones seen in DNA and RNA modifying enzymes that access target bases that are normally not accessible due to secondary and/or tertiary interactions in the nucleic acid molecule. Lastly, the largest limitation in building the model was the number of dispersed binding sites that we observed in our hydroxyl radical data experiment, especially the ones found very distal from the modified nucleotide G1405.

**Binding of ArmA to 30S and SAM**

In spite of the wealth of literature on rRNA methylation, the structural information currently available for RNA methyltransferases is insufficient to elucidate their mechanism of action. Even though directed hydroxyl radical data revealed a very weak and multiple non-specific binding of ArmA to 30S subunit (Figure 22), we were curious to further explore the binding interactions between ArmA and its substrates. Assuming that the absence of SAM does not effect the binding of ArmA to 30S subunit, we decided to determine the affinity of ArmA for each substrate separately. One of the methods used to study the physical basis of molecular interactions between enzyme and substrates is isothermal titration calorimetry (ITC). ITC provides a direct way to the complete thermodynamic characterization of protein interactions. In one ITC experiment we can physically measure the heat generated or absorbed during a binding reaction ($\Delta H_{obs}$). The typical experiment involves addition of one binding partner (titrant) to the other binding partner (titrate) over time using one or more individual injections (Figure 23).
Figure 23. A schematic diagram of a typical isothermal titration calorimeter.
Here, heat is measured as the change in power required for maintaining temperature between the sample and a reference cell. Then, a binding enthalpy is calculated based on knowledge of the cell volume and the concentration of the reactants. The observed enthalpy measured in an experiment includes not only the heat of binding between the molecules, but also any additional heat sources associated with the reaction, including solvent effects, molecular reorganization and conformational changes, and heats of dilution resulting from sample stirring. The heat produced during each injection is proportional to the amount of complex formed, so maximal changes in enthalpy occur at the earliest points in a titration with a decrease in magnitude as free titrant is consumed. Since all equilibrium constants vary with temperature, according to van’t Hoff’s equation (1), we can calculate the affinity constant:

\[
\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2}
\]

(1)

where R is the gas constant, T is the absolute temperature. An equilibrium constant is also related to the Gibbs free energy (\(\Delta G\)) of binding by the reaction (2):

\[
\Delta G = -RT \ln K_a
\]

(2)

Since the \(\Delta H_{\text{obs}}\) is measured directly from the ITC experiment, we can also calculate the reaction entropies (\(\Delta S\)) from the reaction (3):

\[
\Delta G = \Delta H - T\Delta S
\]

(3)

The complete thermodynamic characterization can be obtained from a single ITC experiment, which makes this technique very widely used. With modern ITC instrumentation, sample requirements are greatly reduced, which makes this method applicable to study RNA biochemistry, among other systems. One other benefit of using this method is that it does not require labeling of the protein or substrates, which might
interfere with ArmA folding or binding to the 30S subunit. Because of its benefits and simplicity, we decided to implement the ITC technique to determine the binding of ArmA to its substrate.

First, we titrated ArmA with S-Adenosyl-L-Methionine (SAM). ArmA showed clear binding to SAM, with an affinity in the low micromolar range, 45.8 ± 0.00147 µM. The fit for this titration produced reasonable stoichiometry, with N equal to 1.02 ± 0.138, indicating one SAM molecule bound per protein. The injection profile and the integration (heat release) for each injection (except the first one) are shown in the figure 24. The solid dots indicate the experimental data. The best fit to the experimental data, integrated response versus molar ratio of ligand added, was obtained using a single site fitting model from Origin 7.0 software (OriginLab Corp). The result was in agreement with a Kd value of 18 µM obtained for Sgm, ArmA ortholog, binding SAM\textsuperscript{66,101}. In addition, the binding constant was similar to the Kd value for RsmC, which methylates m\textsuperscript{2}G1207 in 16S rRNA (Kd 4.8 µM)\textsuperscript{102}. It is important to note that K_d\textsuperscript{SAM} is very similar to K_m\textsuperscript{SAM} (40 ±15 µM)\textsuperscript{61}, which indicates that SAM is bound with comparable affinity in the binary complex and in the presence of 30S subunits.

Our next goal was to determine the dissociation constant for ArmA and 30S subunit, since it has not been reported. Since ITC has been successfully used for measuring the binding between the 30S and small molecules\textsuperscript{103}, we assumed that determining the binding of ArmA to 30S subunit would not be any different. However, the absence of any previously reported ITC measurements between a protein and a ribosomal subunit foreshadowed the encountered complications. Only one such (unsuccessful) experiment
has been reported and that was the attempt to measure the binding of the housekeeping methyltransferase RsmC, which methylates m$^2$G1207$^{102}$, to 30S subunit.

The thermodynamics of ArmA – 30S interactions were measured using the VP – ITC 200 calorimeter. Mature 30S subunits were placed in the sample cell and ArmA in the syringe. Both solutions were extensively dialyzed in the same buffer solution containing 40 mM HEPES (pH 7.5), 40 mM NH$_4$Cl, 8 mM Mg acetate, 10% glycerol and 6 mM BME. The concentration of 30S was varied from 2 µM to 10 µM while the ArmA concentration was 10 to 20 times higher than the 30S subunit to insure saturation. Assays were attempted at 20°C or 37°C. The spacing between injections was 300 seconds to insure baseline equilibration after each ArmA sample addition.

Numerous failed trials to measure the binding of ArmA to 30S ribosomal subunits prompted the conclusion that ITC for whatever reason is not suited for this task. Figure 25 shows different isothermal titration calorimetric measurements for ArmA and 30S subunit. From these experiments, we notice that saturation of 30S by ArmA does not occur, even when an excess of ArmA (twenty times) was used to ensure the completion of the reaction. Control experiments verified that the large signals observed were not artifacts. In this case, ArmA was titrated into the buffer (previously described) to ensure that the interaction between ArmA and the buffer does not compensate the heat change between ArmA and 30S subunit. In this experiment, we noticed a very small change in the heat transfer, indicating that the ArmA dilution effect was negligible. We also tested the ArmA – ArmA interaction to ensure that ArmA did not aggregate and cause a large false signal. Once again, we did not observe a large heat change when ArmA was titrated into ArmA.
Figure 24. Isothermal titration calorimetric analysis of SAM binding to ArmA. (A) Titration of SAM (via syringe) into the ArmA protein (sample cell); (B) The binding isotherm fit to a model of a single SAM binding site.
Figure 25. Isothermal titration calorimetric analysis of 30S subunit binding to ArmA. A) Titration of ArmA (via syringe) into the 30S subunits, in ratio 10:1. Figures (A) and (B) depict two different experiments (from different 30S and ArmA preparations)
Therefore, the ITC analysis for ArmA/30S binding appears valid, yet cannot be explained by a simple bimolecular reaction. To ensure the analysis was performed with active ArmA and 30S subunits, both were tested and confirmed active in a standard \textit{in vitro} methyltransferase assay (Figure 26). The 30S subunits were fully methylated in the presence of ArmA and both the enzyme and the substrate were active when the ITC experiments were performed. However, it remains a mystery as to why the 30S/ArmA binding data cannot be simply fit to standard binding models.

To ensure that 30S subunits do not stick to the wall of the sample cell and prevent binding, we reversed the reaction and titrated the 30S into the ArmA (placed in the cell). In this experiment, we varied the concentration of ArmA from 2 to 10 µM and the 30S from 15 to 40 µM. The experiment was performed at 37°C to ensure the same conditions would be followed as for the \textit{in vitro} activity assay. The spacing between injections was 300 seconds to ensure enough time for equilibration. The first titration was 0.5 µL followed by fifteen 2.5 µL injections (Figure 27). Results were identical to those seen in Figure 25, where we did not observe the saturation of ArmA by 30S subunits even when they were in the vast access.

Finally, the question remains of whether or not SAM is required before ArmA can bind 30S subunit. Since SAM could not be used in this experiment (methylation would occur) the product SAH was used, but the data showed the same curve as for the previous experiments (Figure 28A). In the course of conducting the attempt to measure the binding reaction, we also measured the thermodynamic contribution of the methyl transfer reaction. To 30S subunits and SAM placed in the cell, we titrated ArmA and SAM; however, the analysis revealed nearly identical results to all previous ArmA/30S experiments (Fig. 28B).
Figure 26. In vitro methyltransferase activity assay of ArmA. Assays were performed in triplicate; error bars represent standard deviation.
Figure 27. Isothermal titration calorimetric analysis of ArmA titrated into 30S ribosomal subunit.
Figure 28. Isothermal titration calorimetric analysis of 30S binding to ArmA in the presence of SAH (A) and SAM (B).
One possible explanation for the results obtained might be that ArmA does not bind to the 30S subunit very tightly and hence we do not observe the interaction; however, we observed heat changes during the reaction. Another possibility is that ArmA binds to 30S at multiple binding sites, which is consistent with the results obtained from the directed hydroxyl radical probing experiments (Fig 22), thus making it difficult to interpret the binding using the ITC method.

**Fluorescence Polarization Anisotropy**

The second method used to determine the $K_d$ of ArmA and 30S subunit was fluorescence polarization (anisotropy). This method is widely used for measuring the binding interactions between two molecules, and it can be used to determine the binding constant (or the dissociation constant) for various interactions. In this technique, a fluorophore attached to one of the molecules is excited by polarized light. This absorption of light by a population of molecules induces electron passage from the singlet ground electronic level $S_0$ to an excited state $S_n \ (n>1)$. An excited molecule will return to the ground state via emission of a photon (fluorescence), which is measured with a polarizer as a polarized light. Fluorescence polarization is defined by the following equation (4):

$$P = \frac{(I_V - I_H)}{(I_V + I_H)} \quad (4)$$

where $P$ equals polarization, $I_V$ equals the vertical component of the emitted light, and $I_H$ equals the horizontal component of the emitted light of a fluorophore when excited by vertical plane polarized light. However, if the molecule is moving, the polarization of the light will be reduced by radiation in a different direction from the incident light (‘scramble’
This effect is greatest with fluorophores freely tumbling in solution and decreases with decreased rates of tumbling. Upon binding of fluorescently labeled ArmA to the 30S subunit, tumbling of the fluorophore will reduce, thus increasing the amount of polarized light detected. The effect should be particularly large given the relative molecular weight of ArmA (~30,000 D) and the 30S subunit (~900,000 D).

In our experiment, ArmA was fused to fluorescein-5-maleimide at the sulfhydryl-containing cysteine residue. We used the previously described ArmA C115A G234C double mutant for fluorophore attachment at C234, since this residue was exposed to the surface and accessible for labeling. By titrating 30S subunits (from 1 to 2,000 nM) into the 50 nM ArmA, attempts were made to measure changes in polarization, which should be proportional to the amount of ArmA–30S subunit complex formed. However, we were unable to detect the binding of ArmA to 30S over the concentrations tested (Figure 29A).

To determine if the presence of SAM was necessary for ArmA–30S complex formation, we repeated the experiment in the presence of 1 mM Sinefungin (a natural SAM analog). As in the ITC experiments, the presence of a SAM surrogate did not affect the outcome (Figure 29B).

At this point, the question remains open as to the order of substrate addition. Our ITC and fluorescence anisotropy experiments do not confirm that the binding of SAM to ArmA is a prerequisite for ArmA–30S complex formation. The best evidence for that would be the crystal structure of ArmA in complex with its substrates; however, only two structures of methyltransferases, RumA and TrmA, in complex with their respective RNA substrates have been solved\textsuperscript{105,106}. The one unsuccessful attempt to obtain evidence for the nucleotidic substrate binding site for Arm/Rmt group of enzymes was by soaking the
Figure 29. Fluorescence anisotropy for ArmA and 30S in the absence (A) and presence of 1 mM Sinefungin.
RmtB- AdoMet crystals in a solution containing GMP. However, the structure of RmtB – GMP complex revealed that the base and ribose binding sites exactly match those observed for SAM; i.e. the nucleotide G1405 occupies the binding site of the SAM and not the target guanosine in the 16S rRNA.

**Conclusions**

We measured the binding constant of ArmA and its substrate, S- Adenosyl- L-Methionine by ITC method and found the dissociation constant to be 45.8 µM. We were unsuccessful in determining the binding constant of ArmA and 30S subunits using two different methods. It is very possible that the translationally active 30S ribosomal subunits used in our experiments do not bind very tightly to ArmA; hence, we are not able to detect the binding using aforementioned methods. However, the same 30S subunits are capable of being methylated in the presence of ArmA, as shown in our *in vitro* activity assay. This implies that the mechanism of action is complex for this group of enzymes. For that reason we decided to create a minimal kinetic scheme for Arm/Rmt methyltransferases to help us understand the complexity of this group of enzymes (Figure 30). High Mg+2 concentrations stabilize an active state of 30S, while low concentrations of Mg+2 stabilize a translationally inactive conformation of the 30S subunits, represented here as 30S* (k1/k−1 step)69. We have shown (in Chapter 2) that ArmA, in the presence of SAM, can recognizes both forms of 30S subunits and transfer the methyl group to the N7 position of G1405 (k3 step). Once the guanosine nucleotide becomes modified, the product (SAH) is released (k6 step). We took into consideration a possible conformational change in 16S rRNA upon methylation of G1405, which could lead to the formation of different conformation of 30S subunit,
Figure 30. Minimal kinetic scheme of ArmA methylation of 30S subunit. Translationally inactive 30S subunit is represented as 30S*, possible conformation of methylated 30S subunit is represented as me30S**.
represented here as $\text{me}^{3}30S^{\text{**}}$ ($k_{-1}$ step). Since rRNA methyltransferases have not been extensively characterized, one of the experiments to be considered in the future could be to try and trap a more stable ArmA/30S/SAM complex. This way the methylation step would be stalled and the complex would be stable enough to measure the rate of binding. This could be done by having an ArmA mutant which would be catalytically inactive but able to bind to the 30S subunit.

Directed hydroxyl radical probing experiments revealed that ArmA binds very weakly to its substrate, 30S ribosomal subunit. At this time, we are unable to build a model of ArmA – 30S complex that would satisfactorily explain all our footprinting data. Cleavage sites more distal to G1405, even at the opposite face of the ribosome relative to the target nucleotide, suggest that ArmA binds 30S subunits weakly, and possibly non-selectively. Hence, conformational changes that are expected to occur in 16S rRNA upon ArmA binding are most likely complex and involve multiple rearrangements of spatially distant elements that probably have a global character. Data indicate that the mechanism of action for the ArmA group of enzymes is complex and has to be studied in detail by measuring discrete steps of the process. Only when more biochemical data become available will it be possible to design specific inhibitors to treat infections due to bacteria resistant to 4,6-disubstituted aminoglycosides.

**Experimental**

**Mutagenesis**

Primers were designed which contained the mutations of interest flanked by 15-20 bases of wild-type sequence (Table 1). Mutagenesis was performed using the Stratagene
QuikChange Kit according to the manufacturer’s protocol. Briefly, wild-type plasmid was denatured and the primers were annealed and extended by thermal cycling with *PfuTurbo* DNA Polymerase (Stratagene). DpnI (Stratagene) was used to digest the parental (non-mutant), naturally methylated DNA. The plasmids were then transformed into *E. coli* XL1-Blue cells and ArmA mutants were confirmed by sequencing.

**Protein and 30S Subunits Expression and Purification**

Proteins and 30S subunits were expressed and purified as described in Chapter 2.

**Activity Assay**

*In vitro* activity assay was performed as described in Chapter 2.

**Hydroxyl radical probing experiment**

Hydroxyl radical probing experiment was performed under the supervision of Dr. Gloria Culver, as described. In brief, 3 nmol ArmA (cysteineless or cysteine-substituted) were incubated with 70 nmol Fe(II)-BABE in BABE modification buffer (1 M KCl, 80 mM K+ Hepes, pH 7.6, 0.01% Nikkol) in 100 ul total volume at 37°C for 30 minutes. Excess Fe (II)-BABE was removed from derivatized ArmA by centrifugation at 5000 rpm in Microcon (10,000D cutoff) centrifugal concentrators at 4 °C followed by additional washes with 400 µl BABE modification buffer.

**Formation of ArmA/30S subunit complexes**

The formation of the ArmA/30S complex was done essentially as described. In brief, 30pmols submethylated 30S subunits were incubated with 150 pmols Fe(II)-ArmA
in buffer containing 40 mM Tris, pH 7.2, 40 mM NH₄Cl, 8 mM MgOAc, 1 mM DTE in
100 µl total volume at 37 °C for 1 hour. Unbound Fe(II)-ArmA proteins were removed
using Sephacryl S-200 spin columns at 2000 rpm for 3.5 min.

**Primer extension analysis**

Directed hydroxyl radical probing was done essentially as described by Culver and
Noller (2000). Specific ArmA – 30S complexes, 4 µl Fe-EDTA solution, 2 ul 5%
hydrogen peroxide and 2 ul 500 mM ascorbic acid were added in a total volume of 100 ul.
Reactions were performed on ice and quenched by adding 40 µl 0.1 M thiourea. Each
experiment was performed at least three times to insure that data were reproducible.
Extracted 16S rRNA was analyzed by primer extension as previously described by Stern et
al. (1988). Cleavage intensities were compared to the intensity of control sequencing
lanes.

**Isothermal titration calorimetry**

Isothermal titration calorimetry was performed as described. ArmA in 40 mM
HEPES (pH 7.5), 40 mM NH₄Cl, 8 mM Mg acetate, 10% glycerol and 6 mM BME was
titrated against SAM solution prepared by dissolving the appropriate amount of SAM in
the same buffer. The ITC experiment was carried out on a VP - ITC 200 calorimeter
(Microcal, LLC) at 20°C using 0.02-0.1 mM protein in the sample cell and 1-2 mM SAM
in the syringe. All samples were degassed and centrifuged prior to use. Measuring the heat
of dilution for the ligand involved a single 0.5 µL injection, followed by either 1.5 µL or
2.5 µL injection volumes of SAM (depending on the number of injections) with 300
seconds spacing between the injections required for the baseline to equilibrate.
Fluorescence Polarization Anisotropy

ArmA C115A G234C mutant was labeled with Fluorescein 5-Maleimide in the buffer containing 20 mM sodium phosphate, pH 7.5, and 150 mM NaCl. We added EDTA at a final concentration of 5 mM to prevent metal-catalyzed formation of disulfide bonds. We used 25-fold molar excess of Fluorescein 5–Maleimide over the molar amount of ArmA to be coupled. The reaction was allowed to proceed for 30 minutes at 30°C. Non-reacted fluorescein was removed by desalting. The change in polarization was measured by titrating 30S subunits (from 1 to 2,000 nM) into the 50 nM ArmA, using TECAN Polarizon.

Table 1. Primers for Active site mutants

<table>
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<tr>
<th>Mutant</th>
<th>Primer</th>
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<tbody>
<tr>
<td>C115A</td>
<td>5′- GTC TCA TCT ATT TTA GAT TTT GGT GCC GGC TTC AAT CCA TTA GC -3′</td>
</tr>
<tr>
<td>Y43C</td>
<td>5′- GGA AGT TGA GAA TTG CTC TAA AAA GAA ATT GCA TCA AAT ATG G -3′</td>
</tr>
<tr>
<td>Y59C</td>
<td>5′- GTC TTA CTA TTC TGC CTG CCC TAA TTG GGA TAA AIT TTT AAA AAG G -3′</td>
</tr>
<tr>
<td>L65C</td>
<td>5′- CC TAT CCT AAT TGG GAT AAA TGC TTA AAA AAG TAC AAT CAG GGG C -3′</td>
</tr>
<tr>
<td>V190C</td>
<td>5′- CCT GTG CTA AAA CAG CAA GAT TGC AAT TAC ATC TTG GAT TTC CTA C -3′</td>
</tr>
<tr>
<td>M220C</td>
<td>5′- CT GGA AAG GAG AAG GGA TGC GAA GAG AAT TAC CAG CTA TGG TTT G -3′</td>
</tr>
<tr>
<td>G234C</td>
<td>5′- G TTT GAA TCT TTG ACA AAA TGC TGG ATA AAA ATC CTT GAT TCG AAG -3′</td>
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ArmA Methylation of Variant Forms of 30S Ribosome Subunits and 16S rRNA

LiCl Treated 30S Ribosomal Particles

Introduction

In the presence of high concentrations of monovalent salts, such as CsCl, LiCl, or NH₄Cl, the ribosomal proteins dissociate from the 30S subunit producing a series of protein deficient ribosomal particles with different sedimentation coefficients\(^{108}\). This dissociation is primarily the result of a weaker association of proteins on the RNA scaffold due to the suppression of their electrostatic interactions in the presence of higher ionic strength. Both during incubation at a high salt concentration and with stepwise increase in ionic strength, groups of proteins sequentially dissociate from the particles, resulting in the formation of a series of protein-deficient derivatives\(^{108,109}\). In the presence of 1M LiCl, protein-depleted particles sediment as 25S, while higher LiCl concentrations (1.5, 2.0, 3.0 or 3.5 M) produce particles that sediment as 23S, 21S, 19S and 16S, respectively\(^{108}\). The stepwise dissociation of ribosomal proteins from \(E. coli\) 30S subunit with increasing LiCl or CsCl concentration is schematically shown in Figure 31. Initially, at low salt concentration, loosely bound proteins dissociate first, followed by the more strongly bound core proteins. The order of protein dissociation by LiCl or CsCl roughly reflects the order of their assembly into the 30S subunit\(^{13,14}\) (Figure 32).
Figure 31. Scheme of the disassembly of the 30S ribosomal subunit achieved by high concentration of LiCl\textsuperscript{108}. 
Figure 32. *In vitro* assembly map of the 30S subunit. 16S is shown on the top. Arrows indicate interactions between components. The primary, secondary and tertiary binding proteins are colored black, red and green, respectively. The S6 and S18 bind as a heterodimer.13,14.
Results

ArmA recognizes and methylates mature 30S ribosomal subunits, but not phenol-chloroform extracted 16S rRNA\(^{61}\). The question remains as to what the minimal number of ribosomal proteins required for ArmA methylation \textit{in vitro} is. In an attempt to answer this question, 30S ribosomal subunits were prepared by incubation with increased concentration of LiCl using the procedure of Itoh et al (1968)\(^{108}\). The protein depleted particles were isolated using sucrose gradient sedimentation and measured for their methyl acceptor activity. We found that treatment of mature 30S subunits with even low concentrations of LiCl (up to 0.75M) led to a sharp decrease in susceptibility to methylation (Figure 33A). The 30S subunits treated with 1M LiCl showed a very low level of methyl acceptor activity, while no activity was seen for subunits treated with higher concentrations of LiCl. The decrease in ArmA activity is attributed to ribosomal protein loss, since a 2% agarose gel containing formaldehyde confirmed that 16S rRNA was not degraded during preparation of subunits at any of the LiCl concentrations used (Figure 33B).

We further analyzed subunits treated at low concentrations of LiCl (0 to 1M) for changes in the composition of their associated proteins (Figure 34). Since the ArmA activity decreased slightly in the presence of 0.5M LiCl, we sought to identify the ribosomal protein(s) dissociated in this range of LiCl concentration, which are likely to be required for the full activity of ArmA. Qualitative two dimensional acrylamide gel electrophoresis revealed that at low LiCl concentrations (up to 1 M), treated subunits retain the same complement of ribosomal proteins as the LiCl untreated sample, even though their susceptibility to methylation by ArmA drops significantly (Figure 33A).
Figure 33. *In vitro* methylation of LiCl-treated 30S ribosomal subunits (A). A 2% agarose gel containing formaldehyde, confirming that 16S rRNA is not degraded (B)
Figure 34. Two dimensional acrylamide gels showing ribosomal proteins isolated from the LiCl treated particles.
It is important to note that the ribosomal protein S6 was not measured in this 2D gel system, because of its acidic PI\textsuperscript{110}. However, from Figure 31, we note that the ribosomal protein S6 is expected to come off in the higher LiCl concentration and would not influence the decrease in ArmA activity when lower concentrations of LiCl treated particles were used. Even subunits treated with 1 M LiCl, where methylation activity is almost completely abolished, show no significant subtraction of any of the 30S proteins. Although this experiment provides only a qualitative assessment of the protein complement of LiCl treated 30S subunits, the sharp difference in ArmA activity between 0.5 M LiCl and 0.75 M LiCl particles (Figure 33A) in the absence of any detectable difference in protein content (Figure 34) argues against a role for the proteins in the observed loss of ArmA activity.

The dissociation of most of the proteins from the ribosomal particles does not appear to disrupt the overall tertiary structure and compactness of the RNA\textsuperscript{111,112}. Electron microscopy experiments reveal that removing half of the proteins does not lead to significant morphological changes in the particles. When a fragment of the 16S rRNA (corresponding to the central domain) and five proteins S6, S8, S11, S15 and S18 was examined by electron microscopy, the isolated particles had an appearance that was similar in size and shape to the corresponding morphological features of the 30S subunit\textsuperscript{111}. In contrast, our experiments imply that the decrease in ArmA activity is likely due to changes induced by the LiCl in the 16S rRNA. Structural support for the possibility of a significant conformation change in the helix 44/45 region comes from cryo-EM studies of the complex RbfA with 30S ribosomal subunits\textsuperscript{113,114}. To further test our observation, the 30S subunits treated with varying concentrations of LiCl were incubated in high Mg\textsuperscript{2+} buffer
with 50S subunits and showed a progressive decline in the yield of 70S with increasing concentration of LiCl used to treat the 30S subunits (Figure 35). This result, taken with the activity assays for the LiCl treated subunits (Figure 33A), indicates that even at low concentrations of LiCl, only a fraction of the particles are in a conformation that can function as an ArmA substrate.

**Pre-30S Ribosomal Particles as ArmA Substrates**

The 16S rRNA undergoes covalent modification, primarily methylation, at a number of sites during maturation of the 30S ribosome subunit. For the methyltransferase RsmB, protein-depleted 16S rRNA can serve as an *in vitro* substrate. A larger number of methyltransferases, e.g. RsmC, RrmJ, KsgA (RsmA), RsmF, RsmH, and RsmI require a more complex substrate than the naked 16S rRNA for methylation to occur. In the *E. coli* 30S ribosomal subunit crystal structure, many of the nucleotides that are methylated are clearly not accessible to modification enzymes, including those for which the mature 30S subunit serves as a substrate. Some of these may be modified *in vivo* during the maturation process when subunit intermediates offer functional substrates to the modifying enzymes. Others may reflect inherent lability in parts of the 30S subunit that permits transient access to sites that are otherwise sequestered in the most stable form of the assembly. The region of the 16S rRNA with the highest density of nucleotide methylation targets is around the mRNA decoding site, which is known to be structurally dynamic.

It has been shown that the modification at m$^5$C1407 by the housekeeping methyltransferase RsmF is impeded as Sgm, an ArmA homolog, gains access to the nucleotide G1405. In contrast, the two methylations at nucleotide m$^5$C$_{1402}$
Figure 35. Sucrose gradient for 30S LiCl-treated subunits assembled with 50S ribosomal subunits into 70S ribosome. 30S subunit treated with (A) 0M LiCl, (B) 0.5M LiCl, (C) 0.75M LiCl, (D) 1M LiCl assembled into 70S by incubation with 50S ribosomal subunits. Peak number one corresponds to 30S ribosomal subunit; peak number two corresponds to 50S ribosomal subunit; peak number three corresponds to 70S ribosome.
(RmsH and RsmI) are unaffected by the presence of Sgm, indicating a requirement for local changes in the structure of the 30S subunit when these modification enzymes bind to and modify mature 30S subunits, together with a specific order for methylations of nucleotides in 30S subunits.

We tested pre-30S subunits, which do not assemble into 70S ribosomes, as potential ArmA substrates. The pre-30S subunit sediments as fully formed 30S and contains 17S rRNA, a precursor of 16S rRNA, which is the product of the RNAse III cleavage that separates small subunit rRNA from the remainder of the primary transcript (Figure 36). Further processing of 17S rRNA by RNAses E, G, and a still uncharacterized RNase produce the mature 5’ and 3’ ends. Small GTPases Era and RsgA as well as RbfA, RimM, and RimN proteins are required for proper processing of the pre-16S rRNA. The absence of these proteins may impair 17S processing by interfering with 30S assembly and accumulating pre-30S subunits. However, characterization of pre-30S subunits remains incomplete. It is still an open question which ribosomal proteins, housekeeping methyltransferases, and ribosomal binding factors are present in the pre-30S subunits. However, it has been determined that the loss of methyltransferase KsgA alters 16S rRNA processing and accumulation of pre-30S subunits. Even though it has been determined that ArmA recognizes fully formed 30S, we were interested in investigating if the pre-30S ribosomal particles could serve as a substrate.

Fully formed 30S ribosomal subunits were separated from the pre-30S subunits on a sucrose gradient following the procedure of Connolly et al. (2008). For this experiment we used Keio collection cells in which ksgA was deleted (E. coli strain JW0050-3) to
avoid possible inhibitory effects of bound KsgA on ArmA\textsuperscript{76} (see below and Connolly et al. (2008)). The pre-30S subunits were measured for methyl acceptor activity by \textsuperscript{3}H incorporation from \textsuperscript{3}H- SAM (Fig. 37) relative to a control of fully formed 30S ribosomal subunits prepared from 70S ribosomes isolated from the same ∆ksgA strain. Our results show that pre-30S subunits are sub-optimal substrates for ArmA \textit{in vivo}.

Our data imply that the optimal substrate for ArmA \textit{in vivo} is structurally significantly different from the pre-30S subunit. This outcome is not very surprising if we take into consideration that even a small change in 16S rRNA conformation affects ArmA activity and that an almost fully formed 30S subunit is required for the enzyme to transfer a methyl group to G1405.

\textbf{Conclusions}

To determine whether limited depletion of proteins from the 30S subunit affects ArmA methylation activity, we prepared 30S ribosomal subunits by incubation with increasing concentrations of LiCl, using the procedure of Itoh et al. (1968)\textsuperscript{108}. We found that treatment of mature 30S subunits with even low concentrations of LiCl (up to 0.75M) led to a sharp decrease in susceptibility to methylation. The 30S subunits treated with 1M LiCl and sedimenting at 25S\textsuperscript{108} showed a very low level of methyl acceptor activity, while no activity was seen for subunits treated with higher concentrations of LiCl (Figure 33A). This experiment implies that the decrease in ArmA activity is due to changes induced by the LiCl in the 16S rRNA. This is supported by the observation that 30S subunits treated with varying concentrations of LiCl when incubated in high Mg\textsuperscript{2+} buffer with 50S subunits
Figure 36. Schematic of rRNA processing in *E. coli*¹,⁷
Figure 37. *In vitro* methylation of pre-30S subunits and 30S subunits from 70S ribosomes at 37°C and 25°C as ArmA substrates.
showed a progressive decline in the yield of 70S with increasing concentration of LiCl used to treat the 30S subunits (Figure 35). This result taken with the activity assays for the LiCl treated subunits indicates that even at low concentrations of LiCl, only a fraction of the particles are in a conformation that can function as an ArmA substrate.

We also tested whether ArmA can methylate pre-30S subunits, which do not assemble into 70S ribosome. Our results show that these pre-30S subunits are sub-optimal substrates for ArmA in vivo. We conclude that ArmA requires a highly structured ribonucleoprotein particle as a substrate for methylation, and the methylation event in the 3’ minor domain of 16S rRNA occurs late during 30S ribosome assembly.

Experimental

Protein and 30S Subunits Expression and Purification

Proteins and 30S subunits were expressed and purified as described in Chapter 2.

Activity Assay

In vitro activity assay was performed as described in Chapter 2.

LiCl salt washes of 30S subunits

Protein-depleted 30S ribosomal subunits were obtained by treatment with increasing concentrations of LiCl according to Itoh et al. (1968). Briefly, 30S subunits were diluted into buffer (20 mM Tris-HCl, pH 7.8, 100 mM Mg(OAc)$_2$ and 1 mM DTE) at final LiCl concentrations of 0M, 0.5M, 0.75M, 1M, 1.25M, 1.5M, 1.75M, or 2M. Samples with concentrations of LiCl higher than 1M were incubated overnight at 4°C; others were prepared just prior to centrifugation. LiCl-washed particles were pelleted by centrifugation at 45,000 rpm in a Beckmann 70Ti rotor for 6.5h and a sucrose sedimentation profile was
performed as described (Basturea et al, 2007). Pellets were re-suspended in buffer containing 20 mM Tris-HCl, pH 7.8, 0.1 mM Mg(OAc)$_2$, 1 mM DTE and isolated on a 10-30% sucrose gradient by centrifugation in a Beckmann SW28 rotor at 25,000 rpm for 17 hours. Peak fractions were collected, concentrated by centrifugation through an Amicon Ultra 30K cutoff filter, and stored at -80°C.

**Two-dimensional Analysis of 30S and 30S-like Subunits**

The peak containing LiCl-treated RNA-protein complex was also examined on 2D gels, following a protocol derived from Geyl et al (1981) and modified by Lyon and Culver (unpublished data). In brief, LiCl treated subunits were isolated and precipitated using sodium acetate and ethanol. The samples were incubated at -20°C overnight and centrifuged at 10000 RPM for 30 minutes at 4°C. Pellets were then re-suspended in 50µl deionized H$_2$O. Mg-acetate/acetic acid was added to the mixture and centrifuged at 10000 RPM for 30 minutes at 4°C. To the supernatant, 5 volumes of acetone were added, the samples vortexed and precipitated at -20°C, centrifuged at 10000 RPM for 30 minutes and the pellets resuspended in deionized H$_2$O. Samples were run on 2D gels (Biorad) and ribosomal proteins were stained using SYPRO Ruby protein gel stain (Biorad).

Assembly of 70S from 30S LiCl-treated subunits and 50S ribosomal subunits was performed as follows: 30 pmols of 30S subunits (MRE600) from different LiCl treated concentrations (0M, 0.5M, 0.75M, and 1.0M LiCl) were incubated with 60 pmols of 50S ribosomal subunits (MRE600) at 37°C for 1 hour in buffer containing 40mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 100 mM NH$_4$Cl, and 1 mM DTE. Samples were loaded onto a 10-40% sucrose gradient in a buffer containing 40mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 100 mM NH$_4$Cl, and 1 mM DTE and spun in an SW28 rotor at 19,000 rpm for 17 hours.
Competitors for ArmA Binding and Methylation of 30S subunits

The structural basis of aminoglycoside binding to the ribosomal A site has been studied by X-ray crystallography and NMR\textsuperscript{40,45}. The NMR studies, showing the interaction between the aminoglycoside gentamicin and 27 nucleotides that correspond to the \textit{E.coli} 16S rRNA in the region of the A site (Figure 38A), highlighted the hydrogen bond between residue G1405 and the antibiotic. In addition, the crystal structure of an RNA fragment containing the A site (Figure 38B) bound to 4,6- disubstituted aminoglycoside geneticin reveals the detailed interaction between the A site and the antibiotic\textsuperscript{37} (Figure 27C). Results obtained from these studies offer new insights for aminoglycoside - A site complexes. For our studies, it was of particular interest to note that methylation by ArmA will disrupt the hydrogen bond between N7 of G1405 and the 3' amino group of 4,6- deoxystreptamine aminoglycosides and presumably cause steric hindrance between G1405 and ring III of geneticin (Figure 38C). The methylation would also introduce a positive charge on the modified nucleotide, disfavoring the binding of the 4,6- disubstituted aminoglycosides, but not the 4,5- disubstituted group of aminoglucosides, to the decoding site.

It has been observed that translationally active 30S ribosomal subunits are unable to bind 4,6-disubstituted aminoglycoside gentamicin as a result of G1405 methylation\textsuperscript{61}.
Figure 38. The contacts between the A site and gentamicin. (A) The secondary structure of an oligonucleotide that corresponds to *E. coli* 16S rRNA in the region of the A site. The natural sequence is boxed in red; the ArmA modified nucleotide G1405 is shown in red\(^4^5\). (B) Secondary structure of the crystallized RNA fragment\(^4^0\). The two A sites are boxed in red. (C) The contacts between G1405 and 4,6 disubstituted aminoglycoside gentamicin (represented in pink). Hydrogen bonds are shown as dashed black lines.
Since we have shown that ArmA was able to methylate both translationally active and inactive 30S subunits (Chapter 2), we decided to further investigate if the structural changes between the two forms of 30S would extend to the gentamicin binding site. So, 30S subunits were pre-incubated with various concentrations of gentamicin (0.01 mM, 0.1 mM, and 1 mM) in both high [Mg$^{+2}$] (8 mM) and low [Mg$^{+2}$] (4 mM) reaction buffers. After the incubation time, ArmA was added to the solution and *in vitro* ArmA activity assay was measured (Figure 39). The results demonstrate that the presence of gentamicin, even at 0.01 mM, inhibits ArmA activity at both [Mg$^{+2}$] concentrations. The antibiotic appears to bind to the low [Mg$^{+2}$] form slightly better than to the high [Mg$^{+2}$] form (Figure 39), since we observe a greater decrease in ArmA activity at lower concentration of gentamicin in the presence of low [Mg$^{+2}$].

The results were not surprising considering that the inactive 30S conformation (low Mg$^{+2}$) involves structural rearrangement of the 16S rRNA, where some nucleotides become more and some less surface exposed$^{69}$. Changes in reactivity toward probes that were used to monitor the accessibility of pyrimidines at N3 and purines at N-1 and N-7 position in the active and inactive forms of 30S ribosomal subunits have been observed, mostly in the ‘decoding’ A-site at positions 1400 and 1500$^{69}$. In particular, G1494 and U1495 (which are directly involved in gentamicin binding) are more exposed in the inactive conformation of 30S subunits, making them more accessible to the antibiotic. It is also possible that the A site by becoming less rigid in the inactive conformation, allows for easier conformational accommodation upon antibiotic binding. Subsequent direct binding experiments could be performed to test these observations.
Figure 39. Effect of gentamicin on ArmA activity. *In vitro* methylation of wild-type 30S in the presence of low (4 mM) or high (8 mM) Mg$^{2+}$. 
**KsgA Effect on ArmA Activity**

Kasugamycin resistance methyltransferase (KsgA) binds to the 30S ribosomal subunit in the decoding site and along helix 44, even though its sites for methylation (two adjacent adenosines 1518 and 1519) are located in helix 45\(^2\). Since the ArmA modified nucleotide G1405 lies in helix 44 and makes tertiary interactions with A1518, interference and mutual exclusivity of these two enzymes in binding at their target sites would be predicted. Our hydroxyl radical data suggested that some of the binding sites might be close to the KsgA binding sites on the 30S subunit (Figure 22).

For these reasons, we tested whether KsgA inhibits ArmA methylation as a possible indicator of where ArmA binds to the 30S ribosomal subunit. Since KsgA recognizes only the translationally inactive conformation of 30S ribosomal subunit\(^68\), caution needed to be taken when designing the experiment. First, we measured ArmA methylation activity in the presence of KsgA at low (4mM) Mg\(^{++}\) concentration, which stabilizes the translationally inactive conformation of 30S. To ensure that the transfer of methyl group measured in our assay was due to ArmA activity and not KsgA, we used catalytically inactive histidine-tagged KsgA (E66A)\(^117,118\) and 30S subunits prepared from the ksg\(^R\) strain described above. The ksg\(^R\) 30S subunits were pre-incubated with KsgA (E66A) for 10 minutes at 37\(^\circ\)C prior to adding ArmA and \(^3\)H-methyl group incorporation from labeled SAM was measured at intervals over a 2 hour period (Figure 40). Our data show that in the presence of equimolar KsgA, methylation of translationally inactive (low [Mg\(^{++}\)]) 30S subunits by ArmA was reduced by 25%. Furthermore, in 10-fold excess KsgA, ArmA methylation activity was completely abolished.
Figure 40. *In vitro* methylation of 30S. (A) Time course competition assays of ArmA with KsgA E66A. Triangles indicate assays containing 10 pmol 30S ksg<sup>R</sup> 30S and 10 pmols of ArmA; diamonds indicate assays containing 10 pmol 30S ksg<sup>R</sup> 30S, 10 pmols of ArmA, and 10 pmols of KsgA E66A; squares indicate assays containing 10 pmol 30S ksg<sup>R</sup> 30S, 10 pmols of ArmA, and 100 pmols of KsgA E66A; crosses indicate assays containing 10 pmol 30S ksg<sup>R</sup> 30S and 10 pmols of KsgA E66A.
These results correlate well with our prediction that ArmA and KsgA share the same binding site, or at least that their binding sites overlap to some extent. The conclusions of this experiment are qualified by the fact that the lower final counts seen in this in vitro assay might be due to the fact that the translationally inactive 30S subunits lacking the methylation at A1518 and A1519 used in this experiment are not the optimal substrate for ArmA.

**IF-3 effect on ArmA Activity**

Initiation factor 3 (IF3) and KsgA compete for binding to the 30S subunit. Since KsgA and ArmA compete for the same site on the 30S subunit (Figure 40), we extended our competition experiments to determine whether the binding sites of IF3 and ArmA also overlap. We purified histidine-tagged IF3 and used it as a competitor in methylation assays for ArmA activity. Wild type 30S subunits (10 pmols) were incubated with 10 pmols of IF3 in buffer containing 10 mM Mg\(^{2+}\) for 10 minutes prior to addition of ArmA (Figure 41). Our results show that IF3 did not have an inhibitory effect on methylation by ArmA, when compared to the control experiment where IF3 was not present in the reaction. Therefore, we conclude that the common locus for IF3 and KsgA binding does not intersect that for ArmA. Indeed, our hydroxyl radical probing experiment (as well as the footprinting results for Sgm) did not show any cleavage sites at the 790 stem – loop, the most likely site shared by IF-3 and KsgA. To ensure that the complex between IF3 and 30S ribosomal subunit was formed, we used the same condition as for the in vitro activity assay and separated the complex using 10-30% sucrose gradient, which was checked on SDS PAGE gel for the presence of IF-3 (data not shown).
Figure 41. In vitro methylation of 30S. Time course competition assays of ArmA with IF-3. Diamonds indicate assays containing 10 pmol 30S wild type and 10 pmols of ArmA; squares indicate assays containing 10 pmol 30S wild type, 10 pmols of ArmA, and 10 pmols of IF-3.
In vivo analysis

Arm/Rmt activity is insensitive to the methylation status of A1518 and A1519 in vitro (Chapter 2), but it is possible that in vivo, where KsgA has a broader role in ribosome biogenesis beyond that of its methyltransferase function, it could affect Arm/Rmt methylation\textsuperscript{76}. To test for possible other effects of KsgA in vivo, we measured and compared MIC values of the antibiotic gentamicin in wild type and ΔksgA strains of E. coli in the presence and absence of RmtA, which was expressed under the control of an arabinose-inducible promoter (Table 2). (The pBAD plasmid containing the RmtA was kindly provided to us by Matthew Baker). RmtA was overexpressed in the presence of 100 µg/ml ampicillin, 64 µg/ml gentamicin, and either low (0.05%) or high (0.2%) arabinose. If there were no dependence of Arm/Rmt functions on KsgA in vivo, identical MIC values should be seen for the two strains. A lower MIC in the ΔksgA strain, which corresponds to decreased gentamicin resistance, could be due to a partial dependence of RmtA activity on KsgA activity. We found a lower gentamicin MIC for the ΔksgA host under both low and high levels of RmtA (0.05% and 0.2% arabinose concentrations used to modulate RmtA expression). In 0.05% arabinose the ΔksgA strain was more sensitive to gentamicin, with an MIC value of 32 µg/ml versus 256 µg/ml for wild type; similarly in 0.2% arabinose at higher RmtA concentrations the MICs for ΔksgA and wild type were 256µg/ml and 512µg/ml.

However, this observation cannot be ascribed to a direct interplay between KsgA and RmtA, since the control experiment done in the presence of arabinose and the absence of plasmid borne RmtA gave an MIC of 32 µg/ml for the wild type strain and 8 µg/ml in the ΔksgA strain. Therefore, we conclude that there is probably no functional dependence
Table 2. *In vivo* activity assay

<table>
<thead>
<tr>
<th>Cells</th>
<th>Arabinose (%)</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keio-wild type-RmtA</td>
<td>0.2</td>
<td>512</td>
</tr>
<tr>
<td>Keio-wild type-RmtA</td>
<td>0.05</td>
<td>256</td>
</tr>
<tr>
<td>Keio ΔksgA-RmtA</td>
<td>0.2</td>
<td>256</td>
</tr>
<tr>
<td>Keio ΔksgA-RmtA</td>
<td>0.05</td>
<td>32</td>
</tr>
<tr>
<td>Keio-wild-type</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>Keio ΔksgA</td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>
of RmtA on KsgA in vivo under the conditions examined here and that ΔksgA has an inherent increased gentamicin sensitivity arising from other causes.

To further explore the difference in sensitivity between the wild type and ΔksgA strains toward various groups of aminoglycosides, we tested the Keio cells in the absence of RmtA (Table 3). We noticed increased sensitivity toward the 4,6 disubstituted aminoglycosides (tobramycin and amikacin), but decreased sensitivity toward the 4,5 type of aminoglycosides (neomycin and paromomycin). As expected, we did not see a difference in sensitivity between the two strains when streptomycin was used, since this antibiotic has a completely different structure and binding mode.

To test whether RmtA can overcome the higher gentamicin sensitivity of the ΔksgA mutant, we did serial dilutions of E. coli carrying the RmtA plasmid and monitored cell growth for wild type and ΔksgA strains in the presence of 64 µg/ml of gentamicin (Figure 42). At high RmtA (0.2% arabinose), the two strains showed only slight difference in gentamicin sensitivity. However, at low RmtA (0.05% arabinose) we observed a pronounced growth defect in the ΔksgA strain compared to the parental strain. From our experiments, we conclude that RmtA can overcome the inherent increased gentamicin sensitivity in the ΔksgA strain, but only at high levels. Our in vivo studies show that ArmA likely has a relatively narrow temporal window on the ribosome maturation pathway, but a wider window of conformational conditions in which to transfer the methyl group to G1405.
Table 3. MIC towards different aminoglycoside in Keio cells.

<table>
<thead>
<tr>
<th>Aminoglycosides</th>
<th>MIC (Keio wild-type)</th>
<th>MIC (Keio ΔksgA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobramycin (4,6 type)</td>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>Amikacin (4,6 type)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Neomycin (4,5 type)</td>
<td>16</td>
<td>128</td>
</tr>
<tr>
<td>Paromomycin (4,5 type)</td>
<td>16</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 42. Overexpression of RmtA in the wild-type and ΔksgA Keio cells. Plating experiment of saturated culture diluted in ten-fold increments and plated on LB plates containing 100 μg/ml ampicillin, 64 μg/ml gentamicin, and either 0.2% or 0.05% arabinose at 37°C.
Conclusions

We have shown that the differences between active and inactive 30S ribosomal subunit states do not affect ArmA access to G1405 (Chapter 2). This finding was further reinforced by the abolition of ArmA activity in the presence of gentamicin at both high and low [Mg$^{+2}$]. The result obtained here also implies that the gentamicin binding site, in which G1405 lies, is not greatly affected in either low or high Mg$^{+2}$ 30S forms. We also observed a slightly greater decrease in ArmA activity at lower concentration of gentamicin in the presence of low [Mg$^{+2}$], indicating that some very subtle changes in the 30S subunit (between the active and inactive forms) can affect association of the antibiotic with the A site.

We also showed inhibition of ArmA methylation by pre-bound KsgA at low [Mg$^{+2}$], which indicates overlap of the binding sites of KsgA and ArmA. However, this conclusion is qualified by the requirement for some conformational change in 30S subunit for ArmA to access G1405. The conformational excursion for binding both KsgA and ArmA may be the same and each enzyme could be binding to a part of the 16S rRNA which is not accessible in the static, crystallographically determined 30S subunit structure. A similar case is found in the sisomicin-gentamicin resistance methyltransferase Sgm, an ortholog of ArmA, which methylates G1405 in the A site and requires conformational change in order to access the buried target nucleotide$^{66}$.

In contrast, the presence of IF3 did not affect the binding of ArmA to the 30S subunit, indicating that the binding sites for ArmA and IF3 do not overlap. Furthermore, we can conclude that the common locus for IF3 and KsgA binding does not intersect that for ArmA.
Experimental

Proteins and 30S Subunit Expression and Purification

Proteins and 30S subunits were expressed and purified as described in Chapter 2. pBAD/RmtA construct was kindly provided by Matthew Baker.

Expression and purification of mutant E. coli KsgA and IF3

The pET25b-KsgA and pET28b-IF3 constructs were kindly provided by Dr. Heather O’Farrell and Dr. Matthew Hartman of VCU and were confirmed by sequencing. Proteins were expressed in BL21 (DE3) cells (Stratagene) grown at 37°C to an OD<sub>600</sub> of 0.6 in the presence of ampicillin (for KsgAE66A) or kanamycin (for IF3). Protein expression was induced with 0.1 mM IPTG and the cultures grown for a further 4 h. Cells were harvested and broken as for ArmA and KsgAE66A was purified on a HiTrap Ni<sup>++</sup> chelated column equilibrated with 50 mM NaPO<sub>4</sub>, pH 8.0, 300 mM NaCl, 10 mM imidazole. KsgAE66A was eluted with the same buffer containing 250 mM imidazole. IF-3 was purified as previously described<sup>119</sup>. Cells were lysed in 20 mM Tris- HCl, pH 7.7, 60 mM NH<sub>4</sub>Cl, 10 mM MgCl<sub>2</sub>, 10% Glycerol, 5 mM BME, 0.1 mM PMSF and 0.1 mM benzamidine and the cleared lysate loaded onto a HiTrap Ni<sup>++</sup> column. IF-3 was eluted with 20mM Tris-HCl, pH 7.7, 10 mM MgCl<sub>2</sub>, 10% Glycerol, 5 mM BME, 0.1 mM PMSF, 0.5 mM EDTA, and 0.3M imidazole. All proteins were estimated to be >95% pure by SDS-PAGE analysis.

In vitro Activity Assay

*In vitro* activity assay was performed as described in Chapter 2.
**In vivo analysis**

Keio wild type and ΔksgA cells were grown in LB media and ampicillin (100 µg/ml) at 37° C overnight. Saturated cultures were inoculated in LB media in presence of either low (0.05 %) or high (0.2 %) arabinose and ampicillin (100 µg/ml) until they reached OD$_{550}$ of 0.7-0.8. Cultures were diluted in tenfold increments and 5 µl was spotted on LB plates containing 100 µg/ml ampicillin, 64 µg/ml gentamicin, and 0.05% (or 0.2 %) arabinose. Plates were incubated at 37° C overnight.
Conclusions and Future Work

The outlines of *E. coli* ribosome biogenesis were elaborated by Nomura and Nierhaus, but new levels of complexity have been recognized in the overall process in recent years, particularly since the determination of high resolution crystal structures of the ribosome and its subunits and complexes. Transcription and processing of pre-16S rRNA is coordinated with association of both ribosomal and transiently bound proteins, including RNA modification enzymes. While the overall pathway leading to assembly of the mature ribosome is regulated and clearly ordered, there is evidence for parallel sequences of steps that converge downstream in the process.

Bacterial resistance to 4,6-type aminoglycoside antibiotics, which target the small ribosomal 30S subunit, has been traced to the *arm/rmt* family of rRNA methyltransferases isolated from various pathogens. These plasmid-encoded enzymes transfer a methyl group from S-adenosyl-L-methionine to N7 of the buried G1405 in the aminoglycoside binding site of 16S rRNA in the 30S ribosomal subunit. We have characterized aminoglycoside resistance methyltransferase (*armA*), and obtained significant information regarding its mode of action. ArmA methylates mature 30S subunits but not 16S rRNA, 50S or 70S ribosomal particles or isolated Helix 44 of the 30S subunit. To further explore this family of enzymes, we have investigated the
substrate requirements of ArmA. We determined the Mg$^{2+}$ dependence and found that ArmA could recognize both translationally active and translationally inactive 30S ribosomal subunits.

We tested the effects of kasugamycin resistance methyltransferase (KsgA) and initiation factor 3 (IF3) binding to 30S subunits on ArmA activity. The inhibition of ArmA methylation by pre-bound KsgA at low [Mg$^{2+}$] indicates overlap of the binding sites of KsgA and ArmA. However, this conclusion is qualified by the requirement for some conformational change in 30S subunit for ArmA to access G1405. In contrast, IF3 showed no effect on methylation by ArmA, from which we concluded that the common locus for IF3 and KsgA binding does not intersect that for ArmA$^{72}$. We also tested LiCl treated 30S particles and in vivo derived pre-30S ribosomal subunits as ArmA methylation substrates. From these experiments, we conclude that ArmA is most active toward 30S ribosomal subunits that are at or very near full maturation.

However, additional biochemical studies have to be performed in order to fully understand this group of enzymes. Mutations of active site residues would be the first step to explore. This would elucidate the role of residues important for SAM binding and for target guanosine binding. The binding studies could be performed using the ITC method for SAM binding, since we were able to measure the binding of wild-type ArmA and SAM using this technique. Once we identify mutant(s) which are able to bind 30S subunits but are not catalytically active, we could repeat the binding studies for ArmA and 30S subunits. This would help us better understand the complex ArmA mechanism and complete the binding studies for each substrate. Another outstanding question we would like to address involves the order (if any) of substrate binding. It would be of interest to know if the binding of one substrate requires a conformational change of 30S subunits that creates the binding site for the second substrate. These
studies would involve crystallizing ArmA (or its homologs) with both substrates, 30S subunit and SAM. It should be noted that only two structures of methyltransferases in complex with their respective RNA substrates have been determined\textsuperscript{105,106}. Strenuous efforts are therefore required to perform structural studies of ArmA binding to the A site of 30S ribosomal subunits. In addition, it would be interesting to further explore the assembly state of pre-30S ribosomal subunits required for efficient methylation by ArmA. For those experiments, we could use well established in vitro reconstitution assays and in vivo gene knock-out assays to define the structural and assembly state requirements of 30S subunits for ArmA.

All of the aforementioned studies would help us achieve a long term goal – to develop Arm/Rmt specific inhibitors that will not interfere with other physiologically important m\textsuperscript{7}G methylations\textsuperscript{122,123}. Since other m\textsuperscript{7}G methyltransferases\textsuperscript{123} have a different mode of protein-ligand interaction from that of ArmA, the design of inhibitors against the medically important ArmA family of methyltransferases is somewhat simplified. Domains specific for this family of methyltransferases could be considered as targets for particular inhibitors.
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AWARDS
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