Site-specific scission of ribosomal RNA by 110 phenanthroline linked to transfer RNA

Charles C. Rettberg

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Site-specific Scission of Ribosomal RNA
by 1,10 Phenanthroline linked to
Transfer RNA

by
Charles C. Rettberg

Presented in partial fulfillment of the requirements
for the degree of

Master of Science

UNIVERSITY OF MONTANA
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Dean, Graduate School

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Site-specific scission of Ribosomal RNA by 1,10 Phenanthroline linked to Transfer RNA (69 pp.)

Director: Walter E. Hill

ABSTRACT

1,10 phenanthroline linked to position 8 of E. coli transfer RNA$^{\text{Phe}}$ (tRNA$^{\text{Phe}}$) was used to study the interaction of tRNA with ribosomal RNA (rRNA). In the presence of Cu$^{2+}$ and thiols the copper chelator 1,10 phenanthroline cleaves nucleic acids. When linked to position 8 of tRNA$^{\text{Phe}}$ and bound to the hybrid peptidyl/exit site (P/E-site) or exit site (E-site) of the E. coli 70S ribosomes or 50S subunits the tRNA-phenanthroline (tRNA-OP) conjugate cleaves the 23S rRNA at nucleotide 2394. Data are also presented demonstrating that the tRNA-OP structure is equivalent to unmodified tRNA and that the tRNA-OP is not degrading during nucleolysis. These data prove that chemical nucleases can be used as powerful tools for the study of molecular interactions.
Acknowledgements.

For my mother, who always gave me water when I was thirsty.
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ABBREVIATIONS

aatRNA...........amino acyl transfer ribonucleic acid
APM..............[(N-Acryloylamino)phenyl]mercuric Chloride
ATP..............adenosine triphosphate
A-site..........acceptor site of ribosome
cDNA.............complementary deoxyribonucleic acid
cpm.............counts per minute
dNA..............deoxyribonucleic acid
dTT..............dithiothreitol
E.coli..........Escherichia coli
EDTA............ethylenediamine tetra-acetic acid
EF-G............elongation factor G
EFTu............elongation factor Tu
E-site..........exit site of ribosome
fMet-tRNA........formylmethionine transfer ribonucleic acid
GDP............guanosine diphosphate
GTP.............guanosine triphosphate
IF-1............initiation factor 1
IF-2............initiation factor 2
IF-3............initiation factor 3
IOP..............iodoacetal-1,10-phenanthroline
mole..........mole
MPA.............3-mercaptopropionic acid
mRNA.............messenger ribonucleic acid
pmole.........picomole
P-site..........peptidyl site of ribosome
RF-1.............release factor 1
RF-2.............release factor 2
RF-3.............release factor 3
RNA............ribonucleic acid
RRF.............ribosome release factor
rRNA.............ribosomal ribonucleic acid
TC-70S..........tight couple 70S ribosomes
tRNA...........transfer ribonucleic acid
tRNA-OP........transfer ribonucleic acid-phenanthroline molecule
Introduction.

The cellular particle central to protein synthesis is the ribosome. The prokaryotic ribosome of *Escherichia coli* can be dissociated into 30S and 50S subunits. The 50S subunit contains 23S ribosomal RNA (rRNA) 2904 nucleotides in length, 5S rRNA 120 nucleotidase in length, and 32 different proteins designated L1-L32. The 30S subunit contains 16S rRNA 1542 nucleotides in length and 21 proteins designated S1-S21. (Figure 1). Current interest in the ribosome has been spurred on by the discovery of catalytic RNA and it is now believed that rRNA plays an important role in protein synthesis.¹²

1.0 Ribosome Structure and Function.

1.1.0 Ribosomal RNA Processing, Structure, and Function.

1.1.1 Ribosomal RNA Processing.

Although the primary structure of the rRNAs is extremely variable between organisms, there exist regions of primary sequence which are highly conserved phylogenetically. These sequences were the first to be extensively studied for their involvement in translation and are often implicated in the fundamental processes of the ribosome.

As stated above, the *E. coli* ribosome contains three different RNA molecules; the 23S rRNA and 5S rRNA found in the 50S subunit, and the 16S rRNA found in the 30S subunit. The complete sequences of these RNAs were of the first ribosomal RNAs to be elucidated.

The processing of the prokaryotic RNAs involves the
action of multiple ribonuclease enzymes which specifically cleave the 16S and 23S pre-rRNA to form the mature rRNAs. Of those enzymes, ribonuclease III and ribonuclease M16 and M23 are the most important. Ribonuclease III recognizes large stem structures formed by the flanking regions of the 16S and 23S rRNA transcripts and produces specific cleavage sites along this stem. This leaves the mature 16S or 23S rRNA attached to a smaller stem structure which is then cleaved off by the action of either ribonuclease M16 or M23 respectively to form the mature rRNA.\(^3\)

The processing of 5S rRNA involves three different ribonuclease enzymes, RNase III, RNAse E, and RNase M5. Ribonuclease III cleaves the 23S pre-rRNA in the flanking stem region as discussed above and thus releases the pre-5S rRNA for the action of RNase E. RNAse E cleaves the 5S rRNA at specific sites leaving about three nucleotides on both the 3' and 5' end. These nucleotides are the recognized and trimmed off of the mature 5S transcript by RNase M5.\(^4\)

The list of sequenced rRNAs is continuing to grow and now there are several thousand rRNAs from both eukaryotes, and prokaryotes for which sequences are known. By comparing the sequences between organisms, it now appears that other than the highly conserved nucleotides, the primary sequence has little to do with the overall function of the ribosome, and that the overall structure of the rRNAs within the ribosome is what is conserved in evolution.
Figure 1. Model of the 30S subunit, 50S subunit, and 70S ribosome adapted from electron microscopy.
1.1.2 Ribosomal rRNA Secondary Structure.

The secondary structure of rRNA is determined by base pairing of the primary sequence to form single-, double-, and triple-stranded structures. The secondary structures of the E. coli rRNAs are divided into functional domains designated I-III and 3' minor domain for the 16S rRNA, (Figure 2) and I-V for the 23S rRNA. (Figure 3-4). These domains contain both core, and variable regions. The core regions appear to have similar structures in all rRNAs, even though the primary sequences may be vastly different. Phylogenetic comparisons have been the most fruitful technique in determining the secondary structures of the rRNA molecules. By comparing the sequences of the rRNA over a broad range of organisms, a sequence conservation plot is constructed which displays secondary structure and suggests universally conserved sequences which may have functional roles.\textsuperscript{5,6}

By combining phylogenetic analysis with experimental probing, the secondary structure of the rRNA is further resolved. In this case, single or double-strand specific nucleases, chemical probes, or other nucleic acid modifiers are used to either digest or modify the rRNA. Because the modifiers are single-strand specific, double-stranded helices which are suggested by the phylogenetic analysis are immune to digestion or modification and the data can be used to support the phylogenetic findings. The secondary structures of the rRNAs from several organisms are now well characterized using
1.1.3 Ribosomal rRNA Tertiary Structure.

The tertiary structure of the rRNA is formed by the folding of the secondary structure into a three dimensional structure through base pairing, hydrogen bonding, and coaxial stacking. These interactions are further stabilized by interactions with metal ions, and ribosomal proteins. Without crystallographic data the tertiary structure of large RNA molecules is very difficult to determine. Many of the nucleotides involved in tertiary structure are likely to be highly conserved and are difficult to find using a phylogenetic approach. Additionally, intra-rRNA nucleotide interactions are much less common than secondary base pairing, and the interactions between those bases are likely not constrained by classical Watson-Crick base pairing. In fact, in the smaller RNA molecules such as tRNA and several stable RNA hairpin structures in which the three dimensional structure has been deduced by NMR or crystallography, there are a plethora of bizarre intra-RNA interactions including Hoogsteen base pairing and bonding between the bases and the sugar phosphate backbone. In large rRNAs there are probably many of these un-classical bonding patterns which makes solving the tertiary structure much more difficult.

Regardless of those difficulties, several research groups have begun modelling the tertiary folding of the E. coli 5S,
Modelling of the 16S rRNA has received the most attention due to the large amount of data already produced on this molecule, and the 30S subunit. Basically the researchers constrain the structure of the 16S rRNA based on the secondary structure, the proximity to ribosomal proteins, interaction with ribosomal proteins, and RNA-RNA crosslinks. Using these constraints, the general path of the 16S rRNA is traced through the 30S subunit, and the general location of the major domains and helices are known. The efforts of these laboratories have yielded several models of the tertiary folding of the 16S rRNA, but the models are still very general, and far from an atomic picture of the actual structure.

1.2 Ribosomal Protein Structure and Function.

The *E.coli* ribosome contains 55 proteins. The 30S subunit includes 21 proteins designated S1-S21, and the 50S subunit contains 34 proteins designated L1-L34. Except for four proteins, S1, S6, L7, and L12, all the ribosomal proteins are basic and range in size from 9000 to 35000 Da.\(^9\)

Initially, considerable effort in ribosomal research was devoted to the study of the r-proteins. This was largely due to the fact that the techniques for studying proteins at the time were far more advanced than the techniques to study nucleic acids. In addition, catalytic RNA had not been
Figure 2. Secondary structure map of the 16S rRNA from E. coli.
Figure 3. 5' half of the secondary structure map of 23S rRNA from *E. coli*.
Figure 4. 3' half of the secondary structure map of 23S rRNA from E. coli.
discovered and it was therefore thought that the enzymatic activities of the ribosome must be carried out by the proteins. Since that time all of the primary sequences of the *E. coli* r-proteins,\(^{11}\) roughly half of the r-proteins in *S. cerevisiae*,\(^{12}\) and many of the rat-liver r-proteins\(^ {13}\) have been determined. Phylogenetic comparison between the *E. coli* sequences and eukaryotic r-protein sequences has revealed few statistically significant correlations. Only eight proteins of *E. coli* appear to be related to the eukaryotic r-proteins.

The secondary and tertiary structure of some of the ribosomal proteins has been worked on as well, but only two, L6, and L30, have been completely crystallized and the atomic structure determined.\(^ {14,15}\) The structure of the C-terminal end of L7-L12 has also been determined through crystallography.\(^ {16}\) In general, other than the sites of interaction with the rRNA the secondary and tertiary structures of the ribosomal proteins have not been very informative.

As stated above, with a few exceptions, the r-proteins which were once thought to the supply the enzymatic activities of the ribosome have not been found to have many important functional aspects in translation, other than creating the overall structure of the rRNA. Individual functions of some of the r-proteins have been found for only a handful of proteins.\(^ {17,18}\) (Table 1). The lack of functional significance of the r-proteins, however, is also probably a reflection of a lack of study on their structures and functions, and they
are inherently difficult to isolate and characterize.

Although ribosomal research was initiated in the protein field, almost all of the study devoted to ribosomes today is now focused on the rRNAs. The present knowledge of the r-proteins is far from complete and needs much more in depth study to reveal what will most likely be many important functions.

1.3 Ribosome Structure.

Having the ribosomal RNA and ribosomal protein identities fairly well characterized has allowed ribosomal investigators to start modelling the ribosome. The ribosome has been visualized by electron microscopy and the locations of the ribosomal proteins within the ribosome structure have been well studied. Ribosomal proteins have been positioned relative to each other using several techniques. Digestion of the ribosomal subunits with nucleases yield ribonucleoprotein complexes containing several r-proteins. These complexes serve as protein neighborhoods to which data produced by chemical crosslinking can be compared.\(^{19}\)

Another technique used to position the r-proteins relative to each other is immune-electron microscopy.\(^{20}\) Using specific antibodies raised to the individual proteins, the localization of most of the E. coli ribosomal proteins on the subunit or ribosome has been directly visualized. In addition, the antibodies can be used in fluorescent energy transfer
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<td>S1:</td>
<td>Interacts with Initiation Factor 3 (IF3).</td>
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<tr>
<td>S4:</td>
<td>Affects decoding of mRNA; may bind Release Factor 2 (RF2).</td>
</tr>
<tr>
<td>S5:</td>
<td>Affects decoding of mRNA; may bind RF2.</td>
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<tr>
<td>S6:</td>
<td>May interact with termination codons.</td>
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<td>May interact with mRNA.</td>
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<td>S10:</td>
<td>May bind RF2.</td>
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<tr>
<td>S12:</td>
<td>Affects decoding of mRNA; Interacts with streptomycin.</td>
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<td>S18:</td>
<td>May interact with termination codons.</td>
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<td>S21:</td>
<td>Affects mRNA binding.</td>
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<td>Affects tRNA binding in the Exit-site (E-site).</td>
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<tr>
<td>L2:</td>
<td>May interact with termination codons; may be involved with peptidyl transfer.</td>
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<tr>
<td>L7/L12:</td>
<td>May affect GTP hydrolysis rates; binds RF2; interacts with tRNA during elongation.</td>
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<tr>
<td>L10:</td>
<td>May interact with termination codons; affects elongation.</td>
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<tr>
<td>L11:</td>
<td>May bind RF2; affects thiostrepton binding.</td>
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<tr>
<td>L16:</td>
<td>May be involved with peptidyl transfer.</td>
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<td>L27:</td>
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studies to measure the distances between the proteins. But, the most accurate method in determining protein distances has been neutron scattering.\(^{21}\) This technique uses reconstituted ribosomes in which two of the proteins isolated from *E. coli* grown in media containing deuterium. By analyzing the diffraction pattern of the ribosomes containing the deuterated proteins, the relative positions of the proteins to each other can be determined. The protein locations determined by neutron scattering have been the backbone of all the 30S subunit model building to date. Combining the data produced by these techniques has produced models of the topography of the r-proteins in the ribosome.

1.3.1 Small Subunit Structure and Function.

As stated above the *E. coli* 30S subunit contains a 16S rRNA species and 21 different proteins. Once fully assembled the subunit has the dimensions of approximately 220Åx220Åx100Å.\(^{22}\) From electron microscopic observation it appears that the 30S subunit has distinct head, cleft and platform regions. As discussed above, several research groups have begun modeling the structure of the 30S subunit using data obtained from a variety of sources. There are now several working models of the 30S subunit which are constantly undergoing revision.\(^{8,9}\)

Within the 30S subunit the most important structural area appears to be the decoding domain which has been located in
the cleft region as visualized by electron microscopy. This domain contains the last 140 nucleotides of the 3' end of the 16S rRNA as well as proteins S1 and S21. This region of the 16S rRNA has been shown to interact both with the anticodon of tRNA, and with the mRNA through the Shine-Delgarno sequence. Proteins S1 and S21 have also been implicated in IF-3 binding and interaction with the mRNA. It is now theorized that the path of the mRNA may be through the cleft of the 30S subunit where the codon anti-codon interaction between the mRNA and the tRNA takes place. Again, as more data are added to the growing understanding of the ribosome, the models will be progressively more accurate.

1.3.2 Large Subunit Structure and Function.

The *E. coli* 50S subunit contains 34 proteins, 23S rRNA, and 5S rRNA. It has the overall dimensions of approximately 250Å x 250Å x 150Å.\textsuperscript{23} Electron microscopy reveals three major features of the subunit including the L7/L12 stalk, the L1 ridge, and the central protuberance.\textsuperscript{24} In addition, there now appears to exist a large tunnel through the center of the 50S subunit, which may act as a channel for the nascent peptide chain to pass through during translation. The existence of this tunnel, however, is still debated. As with the 30S subunit, the positions of the 50S r-proteins have been established relative to each other, and the folding of the 23S and 5S within the subunit is starting to take shape. The
positioning of the proteins, however, has not been completed by neutron scattering, but through cross-linking and immune electron microscopy. Therefore, the 50S subunit r-protein positions are much less accurate and the working models of the 50S subunit are far behind the resolution of the 30S subunit.

Within the 50S subunit, there appear to be three important structural domains; the GTPase domain, the elongation factor domain, and the peptidyltransferase domain. The GTPase domain is located in the stalk region of the subunit and is associated with nucleotides 1040 through 1100 of the 23S rRNA and proteins L11, and L7/L12. This region is important in binding Elongation Factor-G (EF-G) and is associated with the modulation of the rate of GTP hydrolysis. In addition the nucleotides in the vicinity of 1066 appear to be the site of action for the antibiotic thiostrepton, which acts by inhibiting the binding of EF-G.25

The other elongation factor domain is associated with nucleotides 2650 through 2680 of the 23S rRNA and proteins L3 and L6. This region is placed approximately at the base of the stalk within the body of the 50S subunit. This domain is associated with the binding of elongation factor Tu (EF-Tu) and EF-G. Much study has been focused on this area since it is the site of action for the cytotoxins α-sarcin and ricin.26

Perhaps the most important domain of the entire ribosome is the peptidyltransferase domain.27-29 This domain is associated with nucleotides around 2060, 2450, 2500, and 2600.
as well as proteins L2, L16, and L27. This domain is approximately located at the base of the central protuberance within the body of the 50S subunit. Within this region lies the putative peptidyltransferase center (PT) where the aminoacyl bonds are formed during protein synthesis. There is growing evidence implicating this region as the catalytic core of the ribosome, including crosslinks between the rRNA and 5' end of tRNA,\textsuperscript{30} crosslinks between rRNA and the aminoacyl moiety of tRNA,\textsuperscript{31} and crosslinks between the rRNA and the antibiotic puromycin\textsuperscript{32} which acts by stopping the formation of the growing amino acid chain. This area of rRNA has probably received more attention in structure and function studies of ribosomes than any other, but the actual mechanism in which the peptide bonds are catalyzed by the ribosome is still a mystery.

1.3.3 70S Ribosome Structure.

When the 30S and 50S subunits associate during initiation the 70S ribosome is formed. The \textit{E. coli} 70S ribosome has the approximate dimensions of 170Å×230Å×250Å.\textsuperscript{33} During association the 30S and 50S subunits both undergo conformational changes and contact is made at a specific single region. Above this contact region there now appears to exist a large open area between the subunits in which the components of translation such as mRNA, and tRNA molecules can fit.\textsuperscript{34} This area has been dubbed the interface canyon and is visible in high resolution electron micrographs. Researchers speculate that it is within
this interface canyon that the work of the ribosome is performed. The path of the mRNA and tRNAs during decoding and peptide linkage is presumed to flow through the canyon and through the cleft of the 30S subunit during which time the nascent peptide chain is shunted through the proposed tunnel in the 50S subunit to the cytosolic side of the ribosome. While these images may be very attractive and make common sense, there is little experimental evidence for the path of the translational components during protein synthesis, so most of these suggestions remain in the realm of theory.

It should be noted, however, that since the ribosome is a dynamic particle, the conformation of the rRNA and the proteins is certainly changing during protein synthesis, so many intermediate or transition structures exist. This statement also holds true for the tertiary structure and, to a lesser extent, the secondary structure.

1.4 Translation.

Translation involves the interaction of many cellular components with the ribosome. These include mRNA, tRNA, initiation factors, elongation factors, release factors, GTP, and other many other molecules. The sites of interaction of these components with the ribosome have been investigated using a variety of techniques and the basic stages of protein synthesis have been described, which include initiation, elongation, and termination.
1.4.1 Initiation.

Initiation begins by forming a complex between the 30S subunit, initiation factor-1 (IF-1) and initiation factor-3 (IF-3). (Figure 5). At this point the mRNA is aligned on the ribosome, in part through an interaction between the Shine-Delgarno region of the mRNA and the anti-Shine-Delgarno region of the 16S rRNA, and by interactions with IF-1 and IF-3. A ternary complex of fMet-tRNA/IF-2/GTP then binds to the 30S subunit releasing IF-3. Initiation is completed when the 50S subunit binds, IF-1 and IF-2 are released, and the GTP molecule is hydrolyzed. The initiation complex at this stage contains an interaction of the anticodon of the fMet-tRNA with the AUG start codon of the mRNA in the peptidyl-tRNA binding site (P-site) of the 70S ribosome. (Figure 5).35

1.4.2 Elongation.

Elongation begins with the addition of a tRNA coding for the next codon. This step starts with the association of an elongation factor-Tu (EF-Tu)/GTP/aminocacyl-tRNA (aatRNA) complex with the ribosome. The tRNA is checked for fidelity by the ribosome, with non-cognate tRNA molecules being rejected, and the proper tRNA accepted with the release of EF-Tu/GDP. The ribosome now contains an initiator tRNA in the P-site, and an elongator tRNA in the acceptor tRNA site (A-site). The amino group of the A-site amino acid is then presumably used in a nucleophilic attack on the carbonyl
carbon of the P-site amino acid. The peptide bond is formed, with the subsequent movement of the deacylated tRNA from the P-site to the exit site (E-site) and the elongated peptide covalently attached to the tRNA which is translocated to the P-site.

This translocation of the mRNA and tRNA takes place with the interaction of elongation factor-G (EF-G) and the hydrolysis of GTP. EF-G is then removed leaving the A-site empty and ready for another round of elongation. (Figure 6). The nascent peptide chain is terminated when a stop codon on the mRNA is read. (Figure 6).^{36}

1.4.3 Termination

Termination is performed by release factor-1 (RF-1) or release factor-2 (RF-2) depending on the termination codon and ends in the hydrolysis of the peptidyl-tRNA bond and the release of the peptide from the ribosome. The release of the nascent peptide chain from the ribosome involves the interaction of EF-G and a ribosome release factor (RRF).^{37}
Figure 5. Initiation of translation in *E. coli*. 
Figure 6. Elongation during translation in *E. coli*. 

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1.5 Transfer RNA Processing, Structure and Function.

1.5.1 tRNA Processing.

Pre-tRNA transcripts from *E. coli* are formed either from single genes or from clusters of genes in the vicinity of both mRNA and rRNA transcripts. The mature tRNA is formed through the catalytic action of several ribonucleases. The pre-tRNA transcript is first cleaved by the action of RNase-III leaving a tRNA molecule with approximately 10-40 nucleotides on the 3' and 5' end of the tRNA molecule. RNase-P then cleaves the transcript leaving a mature 5' end. The maturation of the 3' end of the tRNA transcript is not well understood but may include the activities of several ribonuclease enzymes including RNase-F, RNase-PH, and RNase-II among others. After complete maturation of the tRNA transcript many of the nucleotides of the molecule are post-transcriptionally modified by a plethora of enzymes and enzymatic steps. tRNA has perhaps the highest number of modified bases of any nucleic acid characterized, and more than 50 different post-transcriptionally modified bases have been found in the many tRNA molecules which have been fully characterized.

1.5.2 tRNA Structure.

Prokaryotic tRNA molecules vary in length from 72 to 94 nucleotides, but all have a common secondary and tertiary structure. In general all tRNAs have a stem, a D arm, an anticodon arm, a TYC loop, and a variable loop (V-loop).
The most important aspects of this structure are the stem and the anticodon loop. The stem is usually consists of a helical structure formed by base pairing of the 5' and 3' sequences of the molecule. The 3' end of the stem always ends in CCA and it is the site of attachment for the amino acid. The anticodon arm consist of a short helical stem of approximately five base pairs and an open loop of up to seven nucleotides in which the anticodon is located. The nucleotide on the 5' side of the anticodon is always a uridine, and the 5' base of the anticodon is frequently modified, implying a role in its' wobble position.\textsuperscript{41,42}

The tertiary structure of the tRNAs are governed by the same interactions as other RNA molecules, including hydrogen bonding, and coaxial stacking of the individual helices. As mentioned earlier, crystallographic analysis of tRNA has established many non-canonical base pairings within the molecule, including pairings between three base pairs, and hydrogen bonding to the sugar phosphate backbone. Through these bizarre tertiary interactions a common L-shaped feature of all the tRNAs is arrived at, and it is this overall structure which is important for the correct recognition of the tRNA by the corresponding amino-acyl synthetase enzymes, elongation factor-Tu, and the ribosome itself. (Figure 7).

1.5.3 tRNA Function.

The primary function of the tRNA molecule is to shuttle
Figure 7. Three dimensional structure of tRNA from *S. cerevisiae.*
the correct amino acid to the ribosome and present it in the correct conformation on the ribosome so that the peptide bond can be formed. The start of this function is the aminoacylation of the tRNA by its corresponding synthetase enzyme. The aminoacylation of the tRNA molecule is a two-step process in which the amino acid is first made into an aminoacyl adenylate with ATP and is then reacted with the tRNA to form an energy rich acyl bond with the 3' hydroxyl of the terminal adenosine of the tRNA. The amino-acyl tRNA is then either complexed with IF-2 and GTP during initiation or EF-Tu and GTP during elongation. This ternary complex then binds to either the P-site of the 30S subunit during initiation, or the A-site of the 70S ribosome during elongation. At this point peptidyl transfer occurs, GTP is hydrolyzed and the factors are released. The tRNA molecule is then either degraded by nucleolytic enzymes, or recycled back into the system through the synthetase enzyme.

1.6 tRNA-Ribosome Interaction.

1.6.1 tRNA Binding Sites.

Initially it was proposed that the prokaryotic ribosome had only two sites for tRNA binding; the acceptor-site (A-site), and the peptidyl-site (P-site). In the two site model, a round of peptide bond formation begins with an aminoacyl tRNA occupying the P-site of the ribosome, and the A-site being empty. The next tRNA molecule is then shuttled to the
ribosome by EF-Tu and is bound in the A-site followed by the hydrolysis of GTP. Peptide bond formation is then catalyzed by the ribosome, with the transfer of the growing peptide chain to the tRNA located in the A-site. This then causes the release of the deacylated tRNA from the P-site with the subsequent translocation of the tRNA with the growing peptide chain from the A-site to the empty P-site through the action of EF-G and GTP hydrolysis.

It is now accepted that the prokaryotic ribosome has an additional site for tRNA binding called the exit-site (E-site). There are currently two models which include the E-site in the binding of tRNA to the ribosome, the allosteric three-site model, and the hybrid states model.\(^{45-48}\)

The allosteric three site model is similar to the classical two site model, but it utilizes the E-site to bind deacylated tRNA after translocation and before it is released from the ribosome. In this model there are always two tRNA molecules present on the ribosome and both interact with mRNA codons. In addition, it asserts that there is a conformational change which occurs on the ribosome in which in the pre-translational state, the A-site and P-site have a high affinity for binding tRNA with the E-site in a low affinity state, or in the post-translational state, the P-site and E-site are in a high affinity state with the A-site in a low affinity state. In this model, when the A-site is filled, the E-site has a very low binding affinity for deacyl-tRNA. After
translocation, however, the ribosome undergoes a conformational change and the A-site now assume a low affinity binding state with the E-site and P-site binding the deacylated and aminoacylated tRNAs respectively. (Figure 8).\(^4\)

The hybrid states model asserts that during translation the tRNA molecules move through several hybrid binding sites on the ribosome. In this model, the 50S subunit possesses the only site of interaction for deacylated tRNA in the E-site. An aminoacyl-tRNA is initially in the P/P site with the incoming tRNA occupying a hybrid A/entry site. Upon the hydrolysis of GTP and the release of EF-Tu, the two tRNA molecules then rachet to hybrid states consisting of a P/E site, and A/P site. After peptide bond formation, the release of EF-G, and GTP hydrolysis, the tRNA molecules rachet again to occupy the P/P site and the E-site. (Figure 8).\(^4\)

1.6.2 Interaction of tRNA with rRNA.

Chemical modification has shown that a set of highly conserved bases in both 16s and 23s rRNA are protected when either tRNA, or a structural analog of the tRNA is bound.\(^4\) These sites have been the focus for cDNA binding experiments and site specific mutagenesis and several interactions have been substantiated. Perhaps the most convincing evidence for direct tRNA-rRNA interaction comes from crosslinking studies.\(^5\)

Using aryl azide groups attached to different areas of the tRNA molecule, crosslinks to both rRNA and
Figure 8. Allosteric and hybrid states models of tRNA binding in *E. coli.*
ribosomal proteins are formed in situ. These crosslinks confirm a direct interaction between tRNA and rRNA at a resolution of 5-28Å corresponding to the distance between the azide and the tRNA.

From these studies it is clear that the different portions of the tRNA interact with rRNA during translation. The acceptor stem of the tRNA exclusively interacts with the 23S rRNA in several different domains depending on the site of occupation on the ribosome. The anticodon loop exclusively interacts with the 16S rRNA within the decoding domain of the 30S subunit. The sites of interaction as inferred by chemical modification and chemical crosslinking are summarized in Table 2.53

1.7 Techniques to study rRNA.

The size of the prokaryotic ribosome is problematic for the investigator interested in ribosomal structure and function. The ribosome is too small for thorough investigation by electron microscopy yet too large for study by crystallography. In addition there are likely to be many conformations of the active ribosome during protein synthesis further complicating a molecular understanding of the structure. Because of these size limitations many creative and powerful techniques have been adapted for use in the study of ribosome structure.

It is apparent that the ribosomal RNA serves not only as
Table 2. Crosslinking & Chemical Modification Between tRNA & rRNA.

Crosslinking between tRNA and rRNA.

<table>
<thead>
<tr>
<th>tRNA Species</th>
<th>Position in tRNA</th>
<th>Crosslinked Nucleotide</th>
<th>rRNA Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA^ad</td>
<td>34</td>
<td>1400</td>
<td>16S</td>
</tr>
<tr>
<td>tRNA^ag</td>
<td>32</td>
<td>956-986</td>
<td>16S</td>
</tr>
<tr>
<td>tRNA^act</td>
<td>20</td>
<td>1302-1398</td>
<td>16S</td>
</tr>
<tr>
<td>tRNA^agc</td>
<td>37</td>
<td>1533-1543</td>
<td>16S</td>
</tr>
<tr>
<td>tRNA^age</td>
<td>73</td>
<td>1945</td>
<td>23S</td>
</tr>
<tr>
<td>tRNA^agc</td>
<td>32</td>
<td>1338&amp;1378</td>
<td>16S</td>
</tr>
<tr>
<td>tRNA^agc</td>
<td>Amino Acid</td>
<td>2584&amp;2451</td>
<td>23S</td>
</tr>
<tr>
<td>tRNA^act</td>
<td>20</td>
<td>2281-2348</td>
<td>23S</td>
</tr>
<tr>
<td>tRNA^act</td>
<td>46</td>
<td>2308</td>
<td>23S</td>
</tr>
</tbody>
</table>

Chemical Modification of rRNA by tRNA.

<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Modified Nucleotides</th>
<th>rRNA Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-site</td>
<td>529-531, 892, 1405, 1408, 1492-1498</td>
<td>16S</td>
</tr>
<tr>
<td>P-site</td>
<td>532, 693, 794-795, 926, 966, 1338-1339, 1381, 1399-1401</td>
<td>16S</td>
</tr>
<tr>
<td>A-site</td>
<td>2254, 2439, 2451, 2553, 2555, 2602, 2609</td>
<td>23S</td>
</tr>
<tr>
<td>P-site</td>
<td>1916, 1918, 1926, 2252-2253, 2439, 2451, 2505-2506, 2584, 2585, 2602</td>
<td>23S</td>
</tr>
<tr>
<td>E-site</td>
<td>2112, 2116, 2169, 2394</td>
<td>23S</td>
</tr>
</tbody>
</table>
a structural determinant of the ribosome but also is pivotal in many of the functional aspects of protein synthesis and for this reason many of the techniques employed to study ribosomal structure and function are targeted to the rRNA. Chemical modification, mutagenesis, crosslinking, fluorescent energy transfer, and cDNA probing have all been successful to some degree.

1.7.1 Chemical Modification.

Chemical modification allows the researcher to determine the accessibility of each base in a large RNA molecule. Intermolecular and intramolecular interactions are seen as protections or enhancements to specific chemical probes which modify the N1 of purines or the N3 of pyrimidines and are detected by premature termination of reverse transcriptase using the modified rRNA as a template and synthetic DNA oligomers as primers. When ligands are bound to the ribosome the base modifications change and differences at individual bases are interpreted as either direct or indirect interactions between the ribosome and the bound ligand.¹⁴,¹⁵

1.7.2 Chemical Crosslinking.

Crosslinking utilizes highly reactive chemical groups which, when exposed to U.V. light, form covalent bonds to
nitrogens and carbons of both rRNA and ribosomal proteins. These reactive groups are linked to specific sites on molecules which interact with the ribosome. The molecules are then bound in situ on the ribosome and crosslinked. Intermolecular and intramolecular crosslinks are identified as migration differences in two dimensional electrophoresis and the exact location of the crosslink is determined through RNA sequencing.\textsuperscript{56-58}

\textbf{1.7.3 Mutagenesis.}

Site-specific mutagenesis of rRNA allows the researcher to change a single base in the rRNA molecule in order to determine its importance in the structure and function of the ribosome. Using a single stranded vector containing a portion of the rRNA gene, a mismatch oligonucleotide is hybridized and extended. The extension product is then cloned back into a plasmid vector containing the rRNA operon. When a suitable strain is transformed by the plasmid two thirds of the rRNA found in the cell contains the changed base, and the mutant ribosomes can be isolated and studied for changes in their structure and function.\textsuperscript{59-61}

\textbf{1.7.4 cDNA Probing.}

Complementary DNA (cDNA) probing involves binding a complementary DNA probe to single stranded regions of rRNA in
order to perturb function. Single stranded regions of rRNA are the likely candidates for function since they are conserved phylogenetically, and available for interaction. cDNA oligomers are radioactively labeled, bound to the ribosome, and then filtered through a nitrocellulose filter such that only radioactive probe bound to the ribosome is retained on the filter. Probing experiments can elucidate subtle intermolecular interactions as seen by an enhancement or decrease in probe or ligand binding to the ribosome or ribosomal subunit.61-63

1.7.5 Flourescent Energy Transfer.

Fluorescent energy transfer measures the energy transferred between a donor fluorophore in an excited state to an acceptor fluorophore. The energy transfer is dependent on the distance between the donor and acceptor, the orientation of the fluorophores, and the overlap of the emission spectra. With careful controls molecular distances from 30-80Å can be measured.64,66

1.8 Chemical Nucleases.

Although crosslinking studies are very convincing, the methodology is extremely complex and laborious. Many crosslinking reactions have very low yields and can be overlooked by difficult screening procedures. Recently
researchers have discovered a class of metal chelators which can be used in the oxidative scission of nucleic acids. These chemical nucleases, in the presence of a metal cation and a reducing agent, have a nucleolytic activity on both RNA and DNA. A useful metal chelator of this class is the molecule iodoacetal-1,10-phenanthroline (IOP). (Figure 9). IOP is a modified phenanthroline molecule containing a single reactive iodoacetal group which can be covalently linked to proteins or nucleic acids having a thio group.

Since their discovery, chemical nucleases have been used in creative ways to investigate molecular structures and interactions. Sigman first used the nucleolytic activity of 1,10 phenanthroline to investigate the conformation of the lac Operon. In that experiment 1,10 phenanthroline was used as a substitute for enzymatic nucleases to footprint the binding site of the E. coli RNA polymerase on the lac promoter. Use of 1,10 phenanthroline in this study resulted in an increase in resolution of the footprinting due to the smaller size of the phenanthroline molecule compared to the footprinting enzyme.

After it was discovered that 1,10 phenanthroline also cleaved RNA, Nierlich used it along with other footprinting chemicals to determine the lacZ ribosome binding site. In this study it was determined that 1,10 phenanthroline was more evenly reactive and gave a higher resolution than the other chemical probes used.
One of the most interesting studies using 1,10 phenanthroline was done by Helene.\textsuperscript{70} Here the phenanthroline molecule was covalently attached to the 5' end of a DNA probe 11 nucleotides in length which contained the sequence for a target site on Simian virus 40 DNA. Using this approach it was revealed that the phenanthroline-DNA probe conjugate formed a triple helix with the SV40 target DNA and cleaved it.

Another useful chemical cleavage reagent is Iron(II)-Ethylenediaminetetraacetic Acid (EDTA-Fe(II)). Like IOP, EDTA-Fe(II) can be covalently linked to nucleic acids and has been used in the double-strand or sequence specific scission of DNA and RNA. The reaction presumably occurs through an oxidative pathway when reducing agents such as dithiothreitol or sodium ascorbate are added.\textsuperscript{71-72} EDTA-Fe(II) was used by Cech to investigate the tertiary structure of the \textit{Tetrahymena} ribozyme. In that study an EDTA-Fe(II) complex was tethered to guanosine 5'-phosphate and bound into the G-site of the ribozyme. Cleavage of the surrounding nucleic acid structure clarified the three dimensional structure of the catalytic core of the ribozyme.\textsuperscript{71}

More recently, Dervan used EDTA-Fe(II) to investigate the tertiary structure of tRNA\textsuperscript{^he} by covalently attaching the molecule to position 47 and carrying out the auto-cleavage of the modified tRNA\textsuperscript{^he}. The fragments of the cleaved tRNA\textsuperscript{^he} fit closely with the known structure of the tRNA molecule.\textsuperscript{72}

Researchers are still unsure about the exact mechanism in
which a phenanthroline-metal complex cleaves nucleic acids. It was first proposed that cleavage occurred through the release of an oxidative species generated by the reduction of the copper-phenanthroline complex by thiols. More recently researchers have examined the possibility that the phenanthroline is cleaving the nucleic acids by an alkylation of the N-7 group of guanosine followed by depurination and β-elimination. (Figure 9). In either case the cleavage products occur on the opposite strand and are within 2-3 nucleotides of the linked phenanthroline molecule.

1.9.0 Proposed Problem

The footprinting studies, and more importantly, the studies utilizing the nucleolytic activity of 1,10 phenanthroline to investigate the SV40 triple helix and the three dimensional structure of the Tetrahymena ribozyme spurred us to use 1,10 phenanthroline in the study of the molecular interaction between tRNA and the ribosome.

Attaching IOP to a specific site on a tRNA molecule, binding the tRNA-phenanthroline conjugate (tRNA-OP) molecule to the ribosome in situ, and carrying out nucleolysis would allow the rapid and efficient screening of the entire rRNA molecule for intermolecular interaction with tRNA. I investigated this approach by covalently attaching the IOP molecule to the naturally occurring thio-uracil at position eight of E. coli tRNA^phe.
Figure 9. Chemical structure of IOP, and possible reaction mechanisms.
2.0 Materials and Methods.

2.1 Ribosome and Ribosomal Subunit Preparation.

Cells from *E. coli* MRE600 were grown to mid log phase in trypticase soy broth at 37°C and then concentrated using a Millipore Pellicon Cassette system. After concentration the cells were pelleted in a Sorvall GSA rotor for five minutes at 5,000 rpm, and the pooled pellets were then ground with alumina in a mortar and pestle in cracking buffer (10mM Tris-HCl pH 7.4, 15mM MgCl₂, 150mM KCl) for 45 minutes at 4°C. The suspension was centrifuged briefly to remove the alumina and un-lysed cells. The alumina fraction was then washed with buffer, re-centrifuged, and the supernatants pooled. The supernatants were then centrifuged at 50,000 x g for one hour, the pellets discarded, and the supernatant centrifuged at 250,000 x g for three hours. The pellets were resuspended in wash buffer (10mM Tris-HCl pH 7.4, 15mM MgCl₂, 0.5M NH₄Cl) and centrifuged at 50,000 x g for one hour and 250,000 x g for three hours. The pellets were resuspended in wash buffer (10mM Tris-HCl pH 7.4, 15mM MgCl₂, 150mM KCl) to produce tight couple 70S ribosomes or in 30-50 buffer (10mM Tris-HCl pH 7.4, 1.5M MgCl₂, 100mM KCl) to produce 50S and 30S subunits. The resuspended crude ribosomal fractions were then pooled and purified using zonal centrifugation through a 10-38% sucrose gradient in a Beckman Ti14 rotor. The appropriate fractions determined by A₂₆₀ readings were pooled and pelleted and then dialyzed against
~500 volumes of either tc70 or 30-50 buffer for 12-15 hours. Ribosomes or ribosomal subunits were then divided into small aliquots and stored at -70°C.75

2.2 Oligodeoxynucleotide synthesis and purification.

DNA oligomers were synthesized on a Biosearch 8600 DNA synthesizer using standard phosphoramidite chemistry. The oligomers were removed from the support column by incubating the contents in concentrated NH$_4$OH at 55°C for 12 hours. The crude oligomer mixture was then purified using NENprep nucleic acid purification cartridges (DuPont) according to manufacturers' instructions.76

2.3 5'-end Labeling of Nucleic Acids.

2.3.1 Dephosphorylation of Transfer RNA.

Approximately 100 picomoles (pmol) of E. coli tRNA$^{60k}$ was resuspended in 100mM Tris-HCl pH 8.0 and incubated with 1 units of RNase free alkaline phosphatase for one hour at 50°C. The reaction was then extracted three times with equilibrated phenol, ethanol precipitated, and resuspended in PKBM buffer (50mM Tris- HCl pH 7.6, 10mM MgCl$_2$, 3mM DTT, 0.1mM EDTA).77

2.3.2 Phosphorylation.

To approximately 50pmol of DNA oligomer or 50pmol of dephosphorylated tRNA, 10μl of ATPγP$^{32}$ (3000 Ci/mMol) and 2 units of T4 polynucleotide kinase were added in PKBM buffer.
The reaction was incubated at 37°C for 30 minutes. The product was then purified using NENsorb 20 columns (DuPont) according to manufacturers' instructions. The purified fractions were pooled, dried, and resuspended in TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA).

2.4 Iodoacetal-1,10-phenanthroline Attachment to E. coli tRNA<sup>phe</sup>.

Two A<sub>260</sub> units of E. coli tRNA<sup>phe</sup> were dissolved in 35mM TEA-OAc pH 7.6, 3.8M acetonitrile, 0.1mM DTT, and 4.25mM iodoacetal-1,10-phenanthroline (a kind gift from Dr. David Sigman, UCLA) in DMSO, and allowed to incubate at 25°C for 12 hours in the dark. Following the attachment, the tRNA-OP product was precipitated out of the reaction with three volumes of ethanol and resuspended in 15 μl of tracking dye (0.02% bromophenol blue, 0.02% xylene cyanol, 7M urea). The sample was then loaded on a 20% polyacrylamide gel containing 7M urea (dPAGE) in TBE buffer (89mM Tris-borate pH 8.3, 1mM EDTA) and run at 40 mA for one hour. Following electrophoresis the products were visualized using U.V. shadowing and the slowest moving band was cut from the gel and isolated using electro-elution in TE buffer containing 3M Na-OAc in the annode buffer. The elution was run at 5mA for one hour. The isolated product was then ethanol precipitated and resuspended in TE buffer to a concentration of approximately 10pmol/μl as determined by A<sub>260</sub> readings. To quantify the
attachment of the phenanthroline molecule to tRNA the isolated product was loaded on a 20% dPAGE affinity gel containing 10 µg/ml of [(N-Acryloyamino)phenyl] mercuric chloride (APM), and visualized by staining with 0.2% methylene blue. \textsuperscript{79,80}

2.5 Filter Binding Assay.

A mixture of 5'-end labeled and unlabeled tRNA or tRNA-OP having a specific activity of 500 cpm per picomole was bound to un-programmed 70S ribosomes at a 1:1 ratio in binding buffer (6mM MgCl\textsubscript{2}, 10mM Tris-HCl pH 7.5, 100mM KCl). The reaction was incubated at 37°C for 30 minutes and then at 4°C for 3-5 hours at which time the reaction was diluted to 1ml in binding buffer and filtered through a 0.45μm nitrocellulose filter. After repeated buffer washes the radioactivity retained on the filter was counted using liquid scintillation. \textsuperscript{81}

2.6 \textit{In vitro} Protein Synthesis.

A solution containing 17mM Tris-HCl pH 7.4, 17mM Mg-OAc, 68mM NH\textsubscript{4}Cl\textsubscript{2}, 3mM ATP, 3mM GTP, 8mM BME, 3mM DTT, 17mM PEP, and 0.001mM tRNA or tRNA-OP was added to a mixture containing pyruvate kinase, s150 fraction, C\textsuperscript{14} phenylalanine, and tc70S ribosomes. The reaction was incubated for 10 minutes at 37°C at which time poly U was added as message. The reaction was then incubated at 37°C for 30 minutes, stopped with 1ml of ice cold 5% TCA, heated to 90°C for 5 minutes and filtered through
a GC filter. Following several washes with ice cold TCA, the filters were dried and the retained radioactivity was counted using liquid scintillation.

2.7 Nucleolysis of rRNA by tRNA-OP.

tRNA-OP was bound to un-programmed 70S ribosomes at a 1:1 ratio in binding buffer at 37°C for 30 minutes. At the end of the 30 minute incubation 1000 pmol of CuSO₄ and 1000 pmol of MPA was added to induce nucleolysis. The reaction was incubated an additional 3-5 hours at 4°C at which time the rRNA was extracted 3 times with an equal volume of phenol, and ethanol precipitated. The rRNA pellet was then resuspended in 15μl of tracking dye, run on a 5% polyacrylamide-7M urea gel, and visualized by staining with 0.2% methylene blue.

2.8 Sequencing of rRNA.

Ribosomal RNA fragments were visualized using U.V. shadowing, cut from the gel, eluted, and ethanol precipitated as described above. Approximately one microgram of the eluted fragment was annealed to a 5' end labeled primer complementary to a rRNA region 35 nucleotides on the 3' side of the rRNA fragment in annealing buffer (50mM Tris-HCl pH 8.0, 20mM MgCl₂, 100mM KCl). The annealing reaction was heated to 80°C for 3 minutes, quenched on ice, and then divided into four parts for addition to each dideoxy extension mix. Typically 2.5μl of the annealing reaction was added to 3.0μl of the
dideoxy dilution (0.25mM ddA, 0.25mM ddC, 1.25mM ddG, 1.25mM ddT), and 5.0μl of extension mix (0.2mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.2mM dTTP, 50mM Tris-HCl pH 8.0, 125mM KCl, 25mM MgCl₂, 10mM DTT, 0.2 units AMV reverse transcriptase). The extension reaction was incubated at 37°C for 45 minutes at which time 6μl of stop solution (98% formamide, 0.02% xylene cyanol) was added. The reactions were boiled for 3 minutes and loaded on a 0.4mm 14% polyacrylamide-7M urea gel. Electrophoreses was for 3-4 hours at 70 watts constant power and following electrophoresis the gel was visualized using autoradiography.
3.0 Results

3.1 Purification and Testing for Self Cleavage of the tRNA-OP Molecule.

After attachment of the IOP to the E. coli tRNA\textsuperscript{phe} the modified tRNA-OP molecule was purified by gel-electroelution. The tRNA-OP band was eluted from the gel slice using TE buffer containing 3M Na-OAc in the anode buffer. Yields varied from 50-70%.

In order to quantify the attachment of the phenanthroline molecule to E. coli tRNA\textsuperscript{phe}, affinity gel electrophoresis was used. Figure 10 shows the results of running tRNA\textsuperscript{phe}-OP and unmodified tRNA\textsuperscript{phe} on a 20% dPAGE gel containing 10 \( \mu \)g/ml of APM. Under these conditions there is a substantial migration difference between the unmodified tRNA\textsuperscript{phe} and the tRNA\textsuperscript{phe}-OP molecules due to the affinity of APM for uncomplexed thio groups.

Denaturing polyacrylamide gel electrophoresis was also used to test the purity of the final tRNA-OP product, as well as to see if the tRNA-OP molecule was being degraded during the nucleolysis reaction. Figure 11 shows the purified tRNA\textsuperscript{phe}-OP product run next to unmodified tRNA\textsuperscript{phe}. Under the electrophoresis conditions used, a slight migration difference and a broadening of the product band could be resolved from the unmodified starting material. Figure 8 also shows the reaction product after the purified tRNA-OP was incubated with CuSO\textsubscript{4}, and 3-mercaptopropionic acid (MPA) used to induce
nucleolysis. No difference between the purified tRNA-OP and the reacted tRNA-OP could be observed indicating that the tRNA-OP molecule was not being degraded during nucleolysis.

3.2 Binding of tRNA\textsuperscript{phr}-OP and tRNA\textsuperscript{phr}.

The binding of tRNA or tRNA-OP to active, salt washed tc70S ribosomes was assayed using nitrocellulose filtration. Under the filtration conditions used as described in chapter 2, ribosomes are retained along with any tRNA or tRNA-OP bound to the ribosome, while unbound tRNA or tRNA-OP is not retained. The percent binding of the tRNA or tRNA-OP is then calculated as the molar ratio of the tRNA or tRNA-OP retained on the nitrocellulose to the ribosomes. Table 3 details the binding of tRNA or tRNA-OP. tRNA or tRNA-OP was bound to the ribosome at a 1:1 ratio without message to duplicate the same conditions as the nucleolysis reactions. Under these conditions the binding of the tRNA and the tRNA-OP were nearly equivalent. Competition for binding between tRNA and tRNA-OP was also investigated by first binding unmodified tRNA to the ribosome and then carrying out the binding of labeled tRNA-OP as described. Under the conditions used the tRNA or tRNA-OP is binding in either the E and/or P/E-site so the binding represents the cumulative amount of binding in the two sites. Competition did not significantly decrease the binding of the tRNA-OP.
Figure 10. Affinity gel electrophoresis of purified tRNA-OP. 200 pmol of either yeast tRNA\textsuperscript{phe}, \textit{E. coli} tRNA\textsuperscript{phe}, or tRNA-OP were loaded on a 20\% dPAGE containing 10 \(\mu\text{g/ml}\) APM and run for 2 hours at 20mA. Lane 1: yeast tRNA\textsuperscript{phe}. Lane 2: Crude \textit{E. coli} tRNA\textsuperscript{phe}. Lane 3: Purified tRNA-OP.
Figure 11. Purification of tRNA-OP. 200 pmol of either *E. coli* tRNA<sub>abc</sub>, tRNA-OP, or tRNA-OP incubated with CuSO<sub>4</sub> and MPA were loaded on a 20% dPAGE and run for 2 hours at 20mA. Lane 1: Crude tRNA<sub>abc</sub>. Lane 2: Purified tRNA-OP. Lane 3: Purified tRNA-OP incubated CuSO<sub>4</sub> and MPA.
3.3 Protein synthesis using tRNA or tRNA-OP.

The ability of tRNA-OP or tRNA to carry out in vitro protein synthesis was also investigated. Table 3 describes the levels of incorporated C\textsuperscript{14} Phe when either tRNA\textsuperscript{phe} or tRNA\textsuperscript{phe-OP} was used as the source of tRNA in the poly-uracil directed synthesis of poly-Phe. As with the binding data, the level of protein synthesis between the modified and unmodified tRNA molecules was nearly equivalent.

3.4 Nucleolysis of rRNA by tRNA-OP bound to 70S ribosomes.

Figure 12 shows the rRNA extracted after nucleolysis with tRNA-OP. After the nucleolysis reaction as described in chapter 2, a fragment of rRNA migrating with a size of -520 bases, as estimated by RNA size markers, was produced. The fragment was reproducible over a range of magnesium concentrations from 10mM to 25mM MgCl\textsubscript{2} indicating that the fragment was being produced when the tRNA-OP was bound in the E-site or P/E-site of the 70S ribosome. In addition, a competition between pre-bound unmodified tRNA and tRNA-OP attenuated the cleavage product. No apparent cleavage occurs in the control where IOP is in solution.

3.5 Nucleolysis of 23S rRNA by tRNA-OP bound to 50S subunits.

In order to determine whether the rRNA fragment produced by tRNA-OP was originating from the 23S or the 16S rRNA, nucleolysis experiments were performed on 30S and 50S
subunits. Only the 23S rRNA of the 50S subunit was cleaved by tRNA-OP. No cleavage occurred on the 16S rRNA in the 30S subunits programmed with poly U. (Figure 13) The cleavage product produced was the same size as the rRNA fragment generated on 70S ribosomes, and occurred consistently over a range of magnesium concentrations from 10mM to 25mM. This again indicates that fragment was produced by a tRNA-OP molecule bound in the P/E-site or E-site of the 50S subunit.

3.6 Sequencing the 23S rRNA Cleavage Product.

In order to determine the exact location of the site of scission, RNA sequencing was employed. Figure 14 shows the sequence of the 5' end of the rRNA fragment. Under the sequencing conditions a labeled primer complementary to a region ~35 nucleotides on the 3' side of the cleavage site was extended. The 5' sequence is read at the top where the reverse transcriptase fell off the template. The sequence indicates that cleavage was occurring on the 3' side of nucleotide 2394 of 23S rRNA.
Table 3. Binding and protein synthesis of tRNA-OP and tRNA.

**Filter binding assay** tRNA and tRNA-OP were 5'-end labeled using ATPγS and polynucleotidyl transferase. A mixture of labeled and unlabeled tRNA or tRNA-OP having a specific activity of 500 cpm per picomole was bound to TC70S ribosomes at a 1:1 ratio.

**In vitro protein synthesis** Levels of acid precipitable poly-Phe. synthesized in an in vitro protein synthesis reaction using either unmodified tRNA\(^{\text{phe}}\) or tRNA-OP\(^{\text{phe}}\) in the reaction as described in the materials and methods.

**Binding:**

E.coli tRNA\(^{\text{phe}}\): 19.8% ± 2.5%
tRNA-OP: 22.5% ± 3.0%
tRNA-OP Competition: 17.3% ± 5.5%

**Protein Synthesis:**
tRNA\(^{\text{phe}}\): 27691.5 ± 1257.5 cpm C\(^{14}\) Phe. Incorporated.
tRNA-OP: 28760.0 ± 826 cpm C\(^{14}\) Phe. Incorporated.
Figure 12. Nucleolysis of rRNA by tRNA-OP bound to 70S ribosomes.

Lane 1: rRNA extracted from 70S ribosomes. Lane 2: Control reaction in which all the components of the reaction are present except that the 1,10 phenanthroline was not linked to the tRNA and was free in solution. Lane 3: Nucleolysis reaction as described in chapter 2. Lane 4: Competition in which native tRNA was first bound to the ribosome at a 1:1 ratio before the tRNA-OP was added and nucleolysis was started. Numbers indicate the size, in nucleotides, of RNA size markers. (BRL)
Figure 13. Nucleolysis of rRNA by tRNA-OP bound to 50S subunits.

Lane 1: rRNA extracted from 30S subunits. Lane 2: Control reaction on 30S subunits as described for figure 9. Lane 3: Nucleolysis reaction on 30S subunits as described in chapter 2. Lane 4: rRNA extracted from 50S subunits. Lane 5: Control reaction as described for figure 9. Lane 6: Nucleolysis reaction as described in chapter 2. Lane 7: Competition experiment as described in figure 9. Numbers indicate the size, in nucleotides, of RNA size markers. (BRL)
Figure 14. Sequence analysis of the 23S rRNA cleavage product. The rRNA cleavage product was visualized using U.V. shadowing and cut from the gel, eluted, and ethanol precipitated. The sequencing reactions were run as described in the materials and methods. The sequence is read from bottom to top, the uppermost bands corresponding to where the AMV reverse transcriptase fell off the template at the 5' end of the cleavage product.
4.0 Discussion

The experiments presented here and other recent work have proven the usefulness of chemical nucleases for the study of molecular interactions. This study was initiated to determine if chemical nucleases could be used as a potential tool for the study of molecular interactions in translation. Using the chemical nuclease 1,10-phenanthroline attached to the thio-U at position 8 of *E. coli* tRNA^phe^, cleavage occurred at nucleotide 2394 of 23S rRNA when the tRNA is bound in the P/E-site or E-site.

As noted in chapter one, throughout the history of ribosomal research investigators have struggled with the placement of tRNA on the ribosomal subunits, and the ribosome. The two site model of tRNA binding was eventually replaced by the three site model, and now there is evidence for yet another site. A plethora of inventive molecular techniques have been developed to map the positions of the tRNA molecules on the ribosome during various stages of protein synthesis.

Attachment of phenanthroline to the tRNA molecule was determined through affinity gel electrophoresis. (Figure 10). Similar molecules have been attached to tRNA at the thio-uracil position using the same flouro-acetal chemistry and have been quantified at 80-95% attachment, and that is approximately the same level of attachment we see with the IOP molecule.

From previous studies, in which crosslinking agents were
attached to this site in tRNA, it seemed that attaching the phenanthroline molecule to the thio-uracil position of tRNA should not affect the function of the tRNA in a translating system. Our results support those results also. Table 3 shows the binding of P end labeled tRNA-OP was performed to determine the integrity of the molecule compared to unmodified tRNA. Within the error limits the binding of the modified and unmodified tRNA were equivalent.

A competitive binding experiment was then carried out in which un-labeled, unmodified tRNA was used to inhibit the binding of the tRNA-OP by competing for the same ribosomal binding site as the tRNA-OP. Under the binding conditions used, no competition was observed within the range of error. The lack of competition has two possibilities. First the modified tRNA could be binding non-specifically to the ribosome. However, evidence against this is provided by the in vitro protein synthesis data, and by the consistency and homogeneity of the cleavage product produced by the tRNA-OP. The second possibility is that the modified tRNA may have a slightly higher binding constant. This could be resolved by performing a series of binding experiments with both modified and unmodified tRNA and determining the binding constants of the molecules to the ribosome. If the k of the tRNA-OP was higher than that of the native tRNA and exchange was occurring, then competition would not be seen.

Within the range of error the level of protein synthesis
by either the tRNA or tRNA-OP was equivalent. This suggests that the tRNA-OP is being recognized and aminoacylated by the translational apparatus which is the most stringent test for integrity of the modified molecule.

The cleavage of 23S rRNA by the tRNA-OP molecule bound in situ on the 70S ribosome or 50S subunit was very reproducible. The cleavage product was constant over a range of Mg\(^{2+}\) from 10mM to 25mM. Under the higher magnesium concentrations, and in the absence of message, the tRNA is putatively bound in the P/E-site of the ribosome. The exact site of cleavage was located at nucleotide 2394, as determined by RNA sequencing of the cleavage product. This result indicates that the thio-uracil at position 8 of \textit{E. coli} tRNA\(^{phc}\) is within 15Å of nucleotide 2394 of 23S rRNA when the tRNA is bound in the P/E-site or E-site. (Figure 15)

Chemical modification has located a conserved set of nucleotides in both 16S and 23S rRNA which appear to be involved in tRNA binding. Wagner was one of the first researchers to demonstrate a rRNA-tRNA interaction using this technique, demonstrating an interaction between the 1400 region of the 16S and tRNA.\(^4\) This study was later expanded by Noller establishing several new 16S rRNA-tRNA interactions as well as several 23S rRNA-tRNA interactions with the tRNA bound in the A, P, and E-sites. These interactions were primarily located in domain V of the 23S rRNA in or near the loop putatively involved with peptidyl transfer.\(^5\)
Included in these sites is base C-2394 which displayed a protection from the chemical modification by the tRNA molecule bound to the E-site. Although our reaction conditions with high magnesium (25mM) or with the addition of poly-U should bind tRNA exclusively in the P/E-site of the ribosome we have demonstrated that cleavage occurred at the same nucleotide, C-2394. This is reasonable since the E-site interaction in this hybrid state occurs on the 50S subunit.

Noller has produced a model of tRNA binding to the ribosome which involves several intermediate states in translocation. In this model the tRNA molecule moves through a series of hybrid sites including the peptidyl/exit-site (P/E-site) and the aminoacyl/peptidyl-site (A/P-site). At 10mM magnesium the deaclylated tRNA-OP should be bound in the E site, but at 25mM magnesium or with the addition of poly-U the tRNA-OP should be bound exclusively to the P/E-site. In our experiments we observed hydrolysis of nucleotide C-2394 under both of these conditions. This suggests that position 8 of tRNA does not move more than 15Å during the translocation of the tRNA from the P/E-site to the E-site. Another possibility is that nucleotide 2394 is located in a dynamic region of the rRNA which may either move, or act as a fulcrum for the translocation event. In either case, whether the tRNA is in the P/E-site or in the E-site, the 3'-CCA end of the tRNA is presumably interacting with a set of defined nucleotides of the 23S rRNA which form part of the E-site on
Chemical crosslinking of tRNA to the ribosome has been the most important technique demonstrating the interaction of selected rRNA sequences in the binding of tRNA. Several researchers have used a variety of crosslinking reagents linked to the anticodon of tRNA to crosslink 16S rRNA at positions C-1400, 956-986, and 1533-1543. The study was later expanded and two new nucleotides, A-2503 and U-2506, were crosslinked to the tRNA bound in both the P-site and A-site.

With greater relation to our study, crosslinking reagents have been attached to the dihydrouridine loop (D-loop) and the variable loop (V-loop) of the tRNA molecule and crosslinked to 23S rRNA. Podkowinski used an 18Å crosslinker attached to the D-loop to crosslink nucleotides A-2281 and A-2358 in 23S rRNA. In a separate study Mitchell crosslinked the variable loop of tRNA bound in the P-site to nucleotide A-2309 of 23S rRNA.

Although nucleotide C-2394 was not directly crosslinked in any of these studies, there is good agreement of these results with our data. For instance, if the tertiary structural interaction between the nucleotides 2328-2330 (A-U-
G) with nucleotides 2385-2387 (U-A-C) holds true, then the stem loop containing nucleotide 2309 would be placed proximal to the region containing nucleotide 2394. When the tRNA is bound in the P-site it would allow position 8 of the tRNA to be located near nucleotide 2394 concurrently with the D-loop near nucleotide 2358 and the V-loop near nucleotide 2309. Chemical crosslinking studies in which the crosslinking reagent was attached to the same position on tRNA* have not implicated nucleotide 2394 directly. However, in all of these studies the crosslinking reagent has been tethered to a long carbon linker of approximately 25-30Å. This distance is between two to three times the length of the phenanthroline linkage and may account for the difference in the results.

From these results it appears that this region of the rRNA cradles the body of the tRNA and positions the CCA end in the proposed peptidyl transfer site. When two or more tRNA molecules are bound on the ribosome concurrently they must be positioned close together at both the CCA 3'-terminus and the anticodons. There are currently two configurations proposed which would allow for these close contacts between the neighboring tRNA molecules. The R-configuration positions the planes of the two tRNA molecules at 90° relative to each other, and the S-configuration positions the planes of the tRNAs at 270°.

Our results cannot be used to argue in favor of one configuration over the other. Since the tRNA is not degraded
Figure 15. A portion of the 23S secondary structure map in the area of the cleavage site. PT: Indicates proposed peptidyl transfer region. Cleavage is marked with an arrow.
by the nucleolytic activity of the attached phenanthroline, it is probable that the phenanthroline molecule is projecting out from position 8 towards the 3′-terminus. (Figure 16). If, in fact, that is the location of the phenanthroline when attached, then either configuration could be effectively argued since the phenanthroline would be close to the CCA 3′-terminus of the tRNA and the 23S rRNA concurrently and would allow cleavage of nucleotide 2394 in either conformation. If the maximum distance for cleavage of the rRNA by the phenanthroline molecule is taken to be 15-20 Å, then there would be a greater chance of cleavage of the 23S rRNA when the tRNA was in the R-configuration. However, this is just speculation since the exact location of the phenanthroline molecule when covalently attached to the tRNA is not known.

Future direction of this technique should first include physical studies on the structure of the tRNA-phenanthroline molecule for better interpretation of results. More extensive binding and kinetic data would also be valuable to determine competition effects, and show integrity. Primer extension analysis of the entire rRNA molecule under investigation should be undertaken to thoroughly investigate any cleavage sites which may occur below the level of detection of methylene blue dye. Primer extension would yield more information as to the interactions between the tRNA molecule and the ribosome. Finally, by placing the phenanthroline molecule at different positions on the tRNA and binding the
tRNA-phenanthroline complex to specific sites on the ribosome, the position of the entire tRNA molecule when bound to all the binding sites could be mapped.
Figure 16. Most probable orientation of the phenanthroline group in the tRNA-OP molecule.
References


