Assembly of the 30S ribosomal subunit: S15 as the probe

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Assembly of the 30S ribosomal subunit: S15 as the probe

by

Indumathi Jagannathan

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For the Major Program
"Once we understand the biology of *E. coli,*
we will understand the biology of elephant"

- Jacob Monod

This work is dedicated to my grandmother
and parents.
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The aim of this study is to dissect molecular events that occur during the assembly of 30S subunit of the *E. coli* ribosome. The 30S ribosomal subunit is made up of 16S ribosomal RNA (rRNA) and 21 proteins (S1-S21). Crystal structure of the 30S subunit has answered longstanding questions on the three-dimensional organization of its constituents. However, this view does not expose the nature of interesting cooperative movements and conformational changes that occur during the formation of this macromolecular complex. Hence, biochemical and genetic efforts are required to understand these rearrangements.

In this study, we have employed directed hydroxyl radical probing and used one of the ribosomal proteins (r-proteins) S15 as the probe to identify conformational changes that occur in 16S rRNA during different stages of assembly. S15 is one of the proteins that directly interact with the 16S rRNA and it governs the binding of four other proteins during 30S subunit formation. S15 has been converted into a probe by substituting cysteines at unique positions of the protein and attaching Fe(II) to the cysteines to form Fe(II) tethered S15 proteins (Fe(II)-S15). Hydroxyl radicals can be generated from these tethered sites by Fenton chemistry. The “directed” hydroxyl radicals so produced cleave RNA elements that are in proximity to the tethered sites and these cleavage sites are mapped by primer extension.

Using the recombinant *in vitro* reconstitution system and this directed hydroxyl radical probing approach, we have studied the folding of 16S rRNA proximal to S15 during the course of 30S subunit assembly. These studies have revealed protein-dependent conformational changes that occur in RNA environment of S15. Our work suggests that
binding of r-proteins can result in changes that are quite remote from their primary binding site and that assembly of different domains can influence one another.
CHAPTER 1. GENERAL INTRODUCTION

“RIBOSOME……. This seems a satisfactory name and has a pleasant sound”

--- Richard Roberts 1958, Biophysical meeting

Literature Review

Ribosomes: Protein synthesizing machines of the cell. Ribosomes are organelles found in the cytoplasm of prokaryotic cells. They are also found either free or bound to the endoplasmic reticulum, in chloroplasts and mitochondria of eukaryotes. These macromolecular machines are responsible for one of the most fundamental cellular processes, protein synthesis. Ribosomes bind to messenger RNA (mRNA) and assemble proteins according to the genetic code presented by the mRNA. These large and intricate ribonucleoprotein particles (RNPs) are made up of one-third RNA and two-thirds proteins in prokaryotes. The RNA and proteins are organized as two asymmetric subunits with each subunit having highly conserved structure and function across kingdoms. Ribosomes interact with a multitude of substrates and cellular factors during protein synthesis. Thus, for this process to occur with high fidelity, the functional sites on the ribosome need to be flawlessly formed during its biogenesis. Each actively growing *Escherichia coli* (*E. coli*) cell has about 20,000 ribosomes and proliferating cells expend approximately 80% of their energy in ribosome biogenesis. The strong correlation between the rates of ribosome biogenesis and cell growth further underscores the significance of accurately assembled and functional ribosomes.
**Composition of the *E. coli* ribosome.** With the exception of mitochondrial and chloroplast ribosomes, the *E. coli* ribosome is the simplest system available in nature and hence has been used as the benchmark in the following study. An enormous amount of literature is available on its composition and structure including early studies that can be used as the guide to address assembly in this system and to validate the results obtained. In addition, many antibiotics target bacterial ribosomes. Thus, the study of *E. coli* ribosome assembly should provide new insights into the formation of active sites that interact with ligands and reveal the role of different ribosomal components in the process of formation of this RNP.

The *E. coli* ribosome is a 2.5MDa complex with a sedimentation co-efficient of 70S. The large or 50S subunit is composed of two RNA molecules, 23S ribosomal RNA (rRNA) (2902 nucleotides), 5S rRNA (120 nucleotides) and more than 30 proteins, not all of which are unique. The small or 30S subunit consists of one RNA, 16S rRNA (1542 nucleotides) and 21 unique ribosomal proteins (r-proteins) (S1-S21). The large subunit catalyzes peptide bond formation during translation whereas the small subunit is involved in proofreading the codon-anticodon interaction and in the process binds to the messenger RNA (mRNA) and transfer RNA (tRNA). The subunit-subunit interface accommodates three RNA ligands in the A-site, P-site and E-site. Where A-site tRNA carries the incoming amino acid as dictated by the mRNA code, the P-site tRNA has the growing polypeptide chain attached. The E-site is the binding site to which the t-RNA moves to after delivering the amino acid. The 3'-CCA ends of the tRNAs that connect to the growing or incoming amino acid interact with the 50S subunit, where the peptidyl transferase center is located. The anti-codon stem loop of the
tRNA base-pairs with the codon presented by the mRNA bound to the 30S subunit. By monitoring the accuracy of codon-anticodon interaction, the 30S subunit maintains translational fidelity.

**Elucidation of ribosome structures.** In order to understand the mechanism of ribosome action, structural information and insights into the organization of different components are essential. Comparative sequence analysis has been employed to obtain the secondary structures of 16S rRNA, 23S rRNA and 5S rRNA. As this phylogenetic study reveals, 16S rRNA is made up of helices connected to each other by loops and these RNA elements are organized into three major domains: 5', central and 3' major. Two 3' helices constitute the 3' minor domain (Figure 2(a)). The RNAs of 50S subunit have irregular shapes with the 23S rRNA elements arranged in the form of six domains: I-VI.

Three-dimensional structures of the ribosome and ribosomal subunits have been addressed using electron microscopy (EM) and cryo-EM and have yielded low-resolution information including shape of the particles. Recently, tremendous progress has been made in solving crystal structures of the prokaryotic ribosome and ribosomal subunits at high resolution. Thermophile ribosomes have been used to obtain crystals owing to their stability. With high level of conservation between ribosomes from different organisms, information obtained in the case of thermophiles could be extended to understand E. coli ribosome structures. Atomic structures of 50S subunit from *Haloarcula marismortui* and 30S subunit from *Thermus thermophilus (T. thermophilus)* were published in the year 2000. Structure of the 70S ribosome from *T. thermophilus* in complex with mRNA and tRNA was completed at 5.5Å and the path of the mRNA through the 30S subunit
determined\textsuperscript{13}. The structures of the 30S subunit interacting with antibiotics such as tetracycline, hygromycin B and pactamycin\textsuperscript{14} and with initiation factors IF1\textsuperscript{15} and IF3\textsuperscript{16} have also been deciphered.

**Structure of the 30S subunit:** The three major domains: the 5’, central and 3’ major of the 16S rRNA along with a subset of proteins fold into independent structures (body, platform and head) in the 30S subunit. The 3’ minor domain constitutes two helices in the 3’ end including the penultimate stem of the 16S rRNA (Figure 2 (b))\textsuperscript{6:11}.

The body of the subunit contains helices H1-H19 that are packed as a wedge-shape mass of RNA with H6 forming the “spur” at the bottom. R-proteins S4, S5, S8, S12, S16, S17, and S20 form part of this domain among which four (S4, S8, S17 and S20) are primary binders. The platform consists of helical elements H20-H27. Proteins S15, S6/S18 dimer, S11 and S21 bind to this domain. Helices 21, 22 and 23 coaxially stack to form the main part of the central domain. Helix 21 wraps around the 5’ domain and thus connects the body and platform. S8 has binding site in helix 21 but is not considered part of the platform since none of the platform proteins depend on S8 for their binding to this domain. This domain plays an important role in P-site tRNA binding. The head of the subunit formed from the 3’ major domain consists of helices H28-H43 and interacts with mRNA, elongation factor G, A-site tRNA and several antibiotics including spectinomycin. Proteins S2, S3, S7, S9, S10, S13, S14 and S19 bind to this domain among which S7 is the only primary binder. The left of the head tapers off to form the beak of the subunit. The anticodon stem loop of the E-site tRNA is wedged between the head and the platform regions. Helices 44 and 45 constitute the 3’ minor domain. Helix 44, also called the penultimate stem, is the longest helix in the subunit.
and it stretches from the bottom of the head to the bottom of the body. Although r-proteins such as S20 interact with this helix, none of the proteins bind exclusively to this region.

**Structure of the 50S subunit:** The six domains of 23S rRNA and 5S rRNA interlock in a complex manner to result in an asymmetric tertiary structure ("like a jigsaw puzzle") in contrast to independent domain organization in the small subunit. The large subunit is formed with the r-proteins binding to and stabilizing these intricately woven regions. Domain I of the 23S rRNA is made up of helices 1-25 and lies in the back of the subunit. Domain II (helices 25.1 to 46), the largest of the six domains has three protrusions that reach the subunit interface side of the complex. Helices 42 to 44 form the RNA portion of the L7/L12 stalk that is known to interact with elongation factors. The second protrusion is helix 38, which is the largest, unbranched stem in the subunit. It protrudes towards the small subunit between domain V and 5S rRNA. The third one (helices 32 to 35.1) points directly toward and interacts with the small subunit. Domain III (helices 47 to 60), a compact globular domain makes extensive contacts with domain II. Domain IV (helices 62 to 72) forms most of the interface contacts with the 30S subunit. Helix 69 of this domain interacts with the long penultimate stem of 16S rRNA in the small subunit. Domain V (helices 73 to 95), sandwiched between helices II and IV forms the middle part of the 50S subunit and is intimately involved in the catalytic activity of the ribosome. Among the three regions of this domain, the first one forms the binding site for protein L11. The second forms the central protuberance supported by 5S rRNA and domain II. The third region helps stabilize the elongation factor binding elements by extending toward domain VI. The smallest domain of the 23S rRNA, domain VI, has the sarcin-ricin loop (SRL) that is essential for factor
binding. All the nucleotides of 23S rRNA involved in substrate binding, factor binding and catalytic activity are highly conserved.

The large subunit proteins (L1-L30) are distributed uniformly, mostly on the surface and the majority of them interact with two or more rRNA domains. Protein-mediated interactions are extensive between 23S rRNA and 5S rRNA and the non-globular extensions of some of the proteins penetrate the RNA. Thus the 50S subunit proteins stabilize the RNA structure and the relative orientation of adjacent domains. Notably, the sites of functional significance in protein synthesis i.e. the 30S subunit interface and peptidyl transferase active site are devoid of proteins suggesting that RNA is the catalytic unit and proteins stabilize the three-dimensional scaffold of the RNA. However, the polypeptide exit tunnel is surrounded by six proteins suggesting a role for these proteins in protein extrusion.

**Structure of the 70S ribosome:** The 30S and 50S subunits associate via a number of intermolecular bridges to result in the 70S ribosome. The crystal structure of the prokaryotic ribosome from *T. thermophilus* was solved in complex with its functional ligands: mRNA, A-, P- and E-site tRNAs. Among the twelve inter-subunit bridges, the majority of them are RNA-RNA contacts and are located in the center of the subunits. In the case of the small subunit, these contacts are on the platform and penultimate stem bordering the tRNA binding sites. In the large subunit these form a triangular patch near the peptidyl transferase center. RNA elements from domain IV are predominantly involved in these interactions. In contrast, contacts involving proteins are situated in the periphery of the subunits away from the functional sites. S13 in the head and S15 in the platform of the small subunit are the only two proteins contacting 50S subunit. R-proteins L2, L5, L14 and L19 of the large subunit interact
with the 50S subunit. The only protein-protein interaction between the subunits involves S13 and L5.

Certain regions in the two subunits appear to have some conformational differences when associated and isolated. One of the regions that has different conformations is the “spur” of the 30S subunit. The regions that make contacts with the 50S subunit—head, top of the platform and the penultimate stem of the 30S subunit show conformational differences. In the case of the 50S subunit, some of the 23S rRNA elements, including L1 and L11 RNAs, that are disordered in isolation appear stabilized in the 70S ribosome. These observations suggest that rearrangements occur upon subunit association and tRNA binding.

Thus, the crystal structures have answered long standing questions on the three-dimensional organization of these macromolecular complexes and the nature of interactions with various ligands. While the crystal structures reveal an end point of the assembly, they divulge little about the process of building up of the ribosome from its components. Hence, biochemical and genetic efforts are a requisite to understand the dynamics of ribosome biogenesis. Due to its simple composition and known structure, the 30S ribosomal subunit has been used as the model system in the following study to address the complicated process of assembly.

**Discovery of ribosomes and early assembly studies**

**History of ribosomes, a story.** Two important observations led to the discovery of ribosomes. The first one was that bulk of the RNA is located in the cytoplasm. The second was that the protein synthesizing capacity of a cell correlates with its RNA content. Albert Claude in late 1930s, while working with virus-induced tumors, observed particles
that were inactivated by various agents known to affect nucleoproteins and nucleic acids. He also discovered these fractions in chick embryos and found them ubiquitously in a variety of tissues, adult and embryonic, normal and tumorous. He called these particles “small granules” and later “microsomes” (small bodies), a term used to describe the fraction consisting of pieces of endoplasmic reticulum and ribosomes bound or free. Jeener and Brachet isolated these particles from various tissues, showed that they contain RNA, and hypothesized their role in protein synthesis. Using electron microscopy, George Palade confirmed Claude’s observations (Figure 1). He described microsomes as “small particulate component of the cytoplasm.” Palade and Siekevitz showed that the basophilic nature of cytoplasm was due to microsomes that are ribonucleoprotein particles rich in RNA.

In the late 1950s Zamecnik and collaborators demonstrated the essential role of these particles in protein synthesis. They added C\textsuperscript{14} labeled amino acids and ATP to homogenized rat liver cells and were able to detect small amount of protein production. By the process of elimination they established that cellular organelles such as nucleus and mitochondria are not required for protein synthesis whereas microsomes are required. In this process they also identified other cellular components necessary for protein synthesis such as tRNA and enzymes that tether amino acids to tRNA molecules. In 1961, Matthaei and Nirenberg observed that mRNA was required for intensive protein production in cell-free extracts of *E. coli* and hence it was an essential part of the protein synthesizing machinery. The development of this cell-free protein synthesizing system enabled studies to be carried out to understand the role of each of the different components.
In 1958, at the Biophysical society meeting Roberts coined the term “Ribosome” to designate “ribonucleoprotein particles in the size range 20S to 100S” realizing the inadequacy of and the confusion caused by the phrases such as microsome particles. The majority of the studies to understand the structure, composition and assembly of ribosomes were carried out with *E. coli* as the model system owing to its simplicity and high multiplication rate. Systematic work on *E. coli* ribosomes in late 1950s by several groups demonstrated that these organelles consist of two unequal subunits, observed as two sharp boundaries in an analytical centrifuge. The ribosomal subunits were designated 30S and 50S and together they constitute the 70S ribosome. The size was determined by the rate of sedimentation of a particle when it is spun at high speed in ultra centrifuge. The sedimentation co-efficient or the Svedberg units (S) depends on both the size and shape of the complex and therefore the S values of the two subunits are not additive.

The complexity of constitution of ribosomes was brought to light by thorough work done by Waller and Harris. Using starch gel electrophoresis and carboxymethylcellulose chromatography Waller obtained most of the components. The ribosomal proteins were purified by several laboratories and the composition of the subunits determined. Following this, the sequences of 16S rRNA from 30S subunit, 23S rRNA and 5S rRNA from the 50S subunit and sequences of ribosomal proteins were determined.

Since the 30S subunit has been used as the model in the following study of assembly, developments in the field that led to the current understanding of the composition, organization and assembly of the small subunit of *E. coli* ribosome will be discussed in the following sections of this chapter.
**In vitro assembly of the 30S ribosomal subunit.** Preliminary studies in the early 1960s on composition of the small subunit of *E. coli* ribosome revealed that the 30S subunits can be fractionated into inactive core particles (containing 16S rRNA and a subset of small subunit ribosomal proteins) and protein pools (split proteins) \(^{44}\). The core particles were completely converted to intact 30S subunits by mixing the core particles and split proteins together in vitro. The functional integrity of the subunit was ascertained by mRNA and tRNA binding assays. The above studies for the first time demonstrated that functional 30S subunits can be reconstituted in vitro, in addition to allowing the dissection of different components in assembly of the subunit. A major breakthrough occurred in the late 1960s and early 1970s when Nomura and colleagues established that the information necessary for the assembly of the small subunit of *E. coli* ribosome (30S subunit) is encoded within the proteins and RNA. They demonstrated that functional 30S ribosomal subunits can be reconstituted in vitro from naked 16S rRNA and a mixture of the 30S ribosomal proteins (TP30: Total protein derived from 30S subunits) \(^{45}\). Following this they showed that 16S rRNA along with individually purified protein components resulted in functional 30S subunits in vitro \(^{46; \, 47; \, 48}\). The r-proteins were separated by 2-D gel electrophoresis and the nomenclature (S2-S21) is based on their migration on the gel. Where “S” stands for protein from “small” subunit, the number indicates the size of and charge on the protein. For instance, S2 is the largest and S21 is the smallest of the 30S subunit proteins. However, there are a few exceptions like S8 being smaller than S9 and S11 being smaller than S12 and S13. This study also disclosed that r-protein S1 is not required for in vitro reconstitution of functional 30S subunits.

A series of elegant single addition and single omission studies \(^{46; \, 47; \, 48}\) by Nomura and coworkers resulted in the assembly map (Figure 2(c)) which depicts the protein dependencies.
for binding 16S rRNA. Their efforts shed light on the role of different r-proteins in assembly of the 30S subunit. As illustrated in the assembly map, the 30S ribosomal subunit assembles in a highly ordered and hierarchical manner. The primary binding proteins bind to the 16S rRNA independent of other r-proteins and thus likely nucleate RNA folding of each of the three major domains of 30S subunit (head, body and platform). The secondary binding proteins require at least one primary binding protein to be bound in order to interact with the 16S rRNA. The tertiary binding proteins do not bind to the RNA until at least one of the primary and secondary binding proteins are bound beforehand to the growing RNP.

More recently, it has been demonstrated that functional 30S subunits could be reconstituted using recombinant proteins. Each of the ribosomal proteins (S2-S21) was cloned, overexpressed and purified. Along with natural 16S rRNA, the recombinant proteins assembled to form functional 30S subunits. This system paves the way for a diverse and extensive set of biochemical and genetic manipulation to be performed in order to understand the functional role of any particular ribosomal protein of interest and the nature of protein-RNA interactions that constitute the 30S subunit. The recombinant system has been extensively employed in the following studies to address the assembly of 30S subunit.

**Domain organization of 30S subunit.** As evident from the crystal structure, the domains of 16S rRNA along with a specific set of proteins fold into independent structures in the 30S subunit (Figure 2). Most r-proteins have footprints within one domain in turn enabling its RNA elements to fold autonomously.

In the case of the three major domains, it has been shown *in vitro* that an RNA fragment corresponding to a particular domain with its associated proteins tends to form the
RNP with similar morphological features to that found in full 30S subunit. For instance, when the entire set of 30S r-proteins is introduced to RNA fragment corresponding to the central domain of 16S rRNA, it forms an RNP that resembles the platform of the 30S subunit in morphology and protein constitution \(^{50, 51, 52}\). This suggests that each domain assembles independently of the other and within a domain assembly follows an ordered and cooperative pathway.

The independent organization and converging of the domains near the geometric center, next to the tRNA and mRNA interaction sites suggest that the domains are designed to move relative to one another during the process of translation. The head particularly has very few interactions with the rest of the subunit indicating that this region might move during translation consistent with previous studies \(^{53, 54, 55}\).

Reconstitution of 30S subunits by reacting two in vitro transcripts, one corresponding to the 5' and central domains and the other corresponding to the 3' domain, with 30S ribosomal proteins in trans further supports the domain organization of this macromolecular complex \(^{55}\). 30S subunit so formed co-sediments with natural 30S subunits and resembles the small subunit in both morphology and protein constitution. However, these particles show decreased affinity for tRNA suggesting that RNA-RNA contacts or ribosomal protein mediated interactions play a role in accurate assembly of the subunit and the concept of domain organization is likely an oversimplification of an otherwise complex process. The following study demonstrates that proteins binding to one domain have an influence on the folding and orientation of another domain and this synergy is crucial for the 30S subunit to be functional.
RNA-protein interactions in 30S subunit: Footprinting r-proteins on 16S rRNA. Base-specific probes and hydroxyl radical footprinting have been employed to determine the nucleotides of 16S rRNA that are involved in r-protein binding by examining their reactivity towards these chemical agents. Probes such as kethoxal and dimethyl sulfate modify the bases of nucleotides and the hydroxyl radicals cleave the sugar-phosphate backbone. Naked RNA and RNA-protein complex are treated with these probes and analyzed for the modifications. Nucleotides that are either in contact with the protein or undergo a conformational change upon protein-binding show a difference in reactivity (protection or enhancement) towards the probe. This change in reactivity is mapped using primer where a primer complementary to a region downstream to the RNA elements of interest is hybridized and extended using reverse transcriptase (RT). The enzyme while synthesizing the complementary strand stops upon encountering a modified base. In the RNA-protein complex, if the base is better available for modification by the probe due to a conformational rearrangement, the enzyme stalls at the base. This leads to an increase in the accumulation of the cDNA product that can be seen as a darker band on the sequencing gel. However, when RT encounters the base that is less available to the modification reagent owing to its interaction with the protein, it transcribes the complementary strand with lowered tendency to stall and this is indicated by decrease in the band intensity representing the accumulated product. Thus the change in reactivity of nucleotides and backbone towards different probes can be analyzed by the difference in the intensity of the nucleotide bands on the sequencing gel. In the case of hydroxyl radical probing of naked RNA, sugar-phosphate backbone is cleaved almost uniformly throughout the molecule. However, in the presence of r-protein, the RNA elements to which the protein binds get protected from hydroxyl radical attack and
this region of interaction can be seen on the sequence gel as fainter patch. In the following studies, primer extension has been used extensively to monitor conformational changes in the 16S rRNA.

Genetic manipulation of r-proteins and RNA has also aided in dissecting the residues important in making contacts. For instance, the major determinants that are necessary for the interaction of S8 with 16S rRNA were unearthed by inducing random mutations in the protein. This was followed by screening for the mutants that affected the growth of the host cells indicating defect in the function of the protein. RNA recognition site on S7 was determined by introducing point mutations in the protein and analyzing the effect on binding to 3' major domain of 16S rRNA by nitrocellulose binding assays. Substitutions and deletions in the 16S rRNA have been introduced to characterize the nucleotides important to contact the protein of interest. For example, the central domain of 16S rRNA has been subjected to extensive mutagenesis and the effect of such mutations tested on the interaction of S15 that binds to this region. The RNA environment of some of the r-proteins has been monitored by directed hydroxyl radical probing in the context of ribosome or ribosomal subunits. Fe(II) is tethered to different sites in the protein via a cysteine substitution and hydroxyl radicals produced from these sites cleave the RNA backbone proximal to the site of production. The cleavage sites are mapped by primer extension. This technique applied in the past to understand RNA neighborhood of the protein of interest in a static complex has been employed in the following study to understand the conformational changes that occur in the RNA surrounding S15 during the process of assembly.

Prior to the advancements in crystallization of the complete ribosome and ribosomal subunits, minimal RNPs have been formed and structural tools such as NMR and X-ray
employed to understand the nature of interactions between different r-proteins and the RNA. Structures of S15/16S rRNA \(^70\) and S15/S6+S18-16S rRNA \(^71\) complexes gave insights into the nature of RNA-protein interactions. The structure of S8 in complex with its minimal binding site in 16S rRNA was solved by NMR spectroscopy \(^72\). The structure of individual r-proteins solved by NMR and X-ray crystallography shed light on the possible RNA binding elements in these proteins \(^73\); \(^74\); \(^75\). Crystal structures of the 30S subunit have led to the understanding of location of proteins in the full complex \(^6\). RNA-protein contacts are complicated due to the diverse nature of tertiary structures that the RNA can adopt. Apart from giving insights into the end point of assembly, these structural studies have also brought to light the variety ways in which proteins recognize RNA \(^76\).

**Co-transcriptional assembly.** *In vitro* assembly of 30S subunits from its components occurs at 42°C. The temperature dependence of *in vitro* reconstitution was exploited in kinetic footprinting studies performed in the early 1990s to understand the sequential and cooperative nature of assembly. 30S subunits were reconstituted at different temperatures (ice and 42°C) and the nucleotides that underwent differential change in reactivity depending on the temperature were monitored \(^77\). From the earlier footprinting studies, the change in reactivity of nucleotides was attributed to binding of specific proteins. This study has classified r-proteins into different kinetic groups based on the temperature and thus the order at which the r-proteins bind to the growing RNP. The proteins that bind to the 5' and central domains fall into the early binding class and proteins that bind to the 3' domain fall into the mid and late assembly groups indicating that the 5' and central domains start assembling prior to the 3' domain. In support of these observations as inferred from the assembly map,
four of the seven proteins that interact with the 5' domain are primary binders and there are no tertiary binding proteins in this region. This suggests that RNA elements of the 5' region might be the first to fold as soon as transcribed. S7 is the only protein that nucleates folding of the 3' major domain and five of the eight proteins that bind to this domain are tertiary proteins. Thus, it is highly probable that 3' major assembles last. The kinetic footprinting studies therefore reflected upon the polarity of 30S subunit assembly and brought to light the likely co-transcriptional assembly of the 30S subunit.

Further evidence for cotranscriptional assembly was obtained from the study of transcription of rrnB operon (one of the seven units that code for the ribosomal RNAs in E. coli) and the corresponding biogenesis of ribosomes in vivo. T7 RNA polymerase or host RNA polymerase was used for transcription. T7 RNA polymerase transcribes five times faster than the host RNA polymerase and employing the former to transcribe plasmid rrnB operon resulted in accumulation of 50S particles with plasmid-derived rRNA and decrease in the formation of 70S ribosomes. The particles so accumulated were inactive in vitro in translation. However, when the host RNA polymerase was employed to transcribe the rrnB operon in the plasmid, the 30S to 50S ratio and 70S formation was unaffected. These observations suggest that the coordination between transcription of the rRNA and ribosome assembly is essential for the formation of active ribosomes and ribosomal subunits. This study sheds light on the significance of coupling between the processes of rRNA transcription and ribosome assembly.

**Primary binding r-protein S15 and its RNA ligands.** Among the r-proteins, primary binding proteins are of particular interest since they bind to the RNA independently
and hence by directing the folding of the RNA initiate assembly of the subunit. Some of the r-proteins also bind to the mRNA product of their operon in the process of autoregulating their translation and thus maintaining their levels of expression in cells. R-protein S15, a 10kDa protein with 88 amino acids is one of several proteins that bind specifically to 16S rRNA as well as its mRNA. It has been shown that S15 is one of the bridges between the 30S and 50S subunits by virtue of its contact with an element of 23S rRNA in the 50S subunit. Hence, this protein plays a key role in regulating important cellular processes by virtue of its interaction with its three RNA ligands. Interestingly, the three RNA ligands of S15 share no obvious structural homology. Therefore, functionally dissecting these diverse RNA-protein interactions would give insights into number cellular processes including 30S ribosomal assembly, subunit association and translational regulation.

Conformational variability of S15. The structure of S15 alone has been solved by NMR and X-ray crystallography (Figure 3). Although these structures agree that S15 is a four helical bundle and the overall topology revealed by the two structures is similar, there are significant differences in two regions. In the NMR structure where the N-terminal helix is aligned with rest of the protein, X-ray structure reveals that it is positioned away from the other helices to make contacts with symmetry related molecule in the crystal. This indicates that the helix I has conformational variability and the loop LI connecting helix I to the body of the protein has inherent flexibility. The other difference is in the conformation of helix 3 and loop LII. Both these regions are highly ordered and helix 3 is continuous in the crystal structure. However, helix 3 is broken into two helices and loop LII is disordered as determined by NMR. It has been hypothesized that this observed variation might be the result
of conformational change occurring in the protein upon RNA binding. Although, conformational rearrangement that occur in the 16S rRNA and mRNA upon S15 binding have been studied, little is known about the conformational change that can possibly occur in the protein as a result of binding to the RNA. It has been suggested S15 might adopt different conformations in binding to its different types of RNA ligands. This has been addressed using FRET and this work is currently ongoing.

**Interaction of S15 with its RNA ligands**

**Interaction of S15 with 23S rRNA in 50S subunit.** Subunits are connected by a complex set of molecular interactions. Earlier crystal structure of the 70S at 7.8 Å indicated that majority of them are RNA-RNA contacts. One of the few protein-RNA interactions involves S15 forming a part of the contact of the platform of 30S with double helical bridge that projects from the 50S subunit. The proximity of 23S rRNA to S15 in 70S ribosome was analyzed by performing directed hydroxyl radical probing of 70S reconstituted with Fe(II)-S15 proteins. This work indicated that the 715 loop of 23S rRNA was involved in the interaction with S15 during subunit-subunit interaction and the finding was further confirmed by higher resolution crystal structure of the 70S ribosome at 5.5 Å. However, S15 doesn’t directly bind to naked 23S rRNA or the 18-nucleotide RNA region of the 23S rRNA which forms the S15 binding site in the context of 50S subunit.

**Interaction of S15 with its mRNA.** Earlier work has revealed that *E. coli* S15 controls its own translation by binding to the mRNA at a region that overlaps with the ribosome binding site. Extensive studies involving mutational analysis of the RNA,
footprinting and toeprinting experiments have shed light on the interaction of S15 with the mRNA.

*RpsO*, the gene encoding S15, is part of a polycistronic operon along with the non-ribosomal gene, polynucleotide phosphorylase. Although each of the genes is associated with a promoter and terminator, regulation of their expression is quite complex. In the early 1980s it was proposed that when the concentration of a regulatory r-protein is higher than that of its target site on the rRNA, the protein binds to its own mRNA at the “operator” region and stops translation\(^7\). When *rpsO* was introduced into the cells on a multicopy plasmid, it was observed that the rate of mRNA synthesis increased but that of S15 synthesis did not\(^8\). This suggested that S15 regulates its translation but not transcription which is in agreement with the hypothesis. Location of the operator site was determined by mutational analysis of the rRNA to be at the 5’ non-coding region of the RNA that overlaps with the ribosome binding site. An *in vitro* synthesized mRNA fragment corresponding to *E. coli* rpsO mRNA containing the operator region was monitored with chemical probes and RNase\(^8\). It was shown that this RNA folds into two mutually exclusive conformations that are in dynamic equilibrium: a structure with three hairpins or a pseudoknot with one hairpin. *E. coli* S15 preferentially binds to the pseudoknot and stabilizes this structure. This is indicated by loss of translational control resulting from mutations that disrupt or alter the formation of the pseudo-knot. Toeprinting analysis\(^8\) brought to light that S15 doesn’t inhibit binding of the 30S subunit to the mRNA during translation. However, the r-protein prevents the formation of a functional ternary 30S-mRNA-tRNA\(_{\text{fMet}}\) complex and thus inhibits translational initiation. Titration experiments and competition kinetics suggested that addition of 16S rRNA suppressed the ability of S15 to inhibit the formation of the active ternary complex.
Thus, the rRNA might compete for binding to S15 and thus act as an anti-repressor\textsuperscript{87}. Base-specific and hydroxyl radical probing along with computer modeling of the S15-pseudoknot interaction pointed out that S15 makes contacts with the deep groove of the coaxial stack. In binding so, the protein contacts with both stems, shielding the bridging adenine\textsuperscript{88}. Extensive mutational analysis also suggested the involvement of the wobble base pair U.G in recognition of the pseudoknot by S15 and thus the process of autoregulation. The findings from these experiments further suggested that a unique motif made of two adjacent base pairs, U.G/C.G is vital for translational control\textsuperscript{89}. This implies that the only similarity between the rRNA and mRNA targets is the G-U/G-C motif that is the secondary binding site in the rRNA but is essential for S15 binding to its mRNA. Structural analysis of the minimal mRNA binding site of S15 (containing 45 nucleotides) showed that the G-U/G-C motif recognition occurs from the minor groove in both the RNA targets reflecting on the similarity. It also suggested in contrast to previous reports that U.G most likely contributes to the precise conformation of the RNA to direct binding and might not be contacted by S15.

However, studies of interactions of S15 with its mRNA in \textit{Bacillus stearothermophilus} (\textit{B. stearothermophilus}) using deletion mutants and native-gel mobility-shift assays suggest that S15 binds to a three-way helical junction formed by the mRNA\textsuperscript{90}. Although this region doesn’t share high degree of homology with 16S rRNA, its secondary structure is similar to the binding site of S15 in 16S rRNA. A similar phenomenon was observed in recognition of its mRNA by S15 in \textit{T. thermophilus}\textsuperscript{91}. However, in this case, the secondary site of recognition contains a G-G mismatch flanked by G-C base pairs in place of the G-U/G-C pair. In addition, the mechanism of translational control was reported to be different in these thermophiles. S15 in \textit{T. thermophilus} competed with the ribosome to bind
to its mRNA (displacement model) as against the entrapment model in *E. coli*. Given the high degree of conservation of S15 and rRNA these results are conflicting. It is yet to be determined whether S15 binding sites in 16S rRNA and mRNA are similar. Directed hydroxyl radical probing that has been employed in the current study can be extended to understand the secondary structure of mRNA which S15 recognizes and thus uncover the mystery.

**Interaction of S15 with 16S rRNA in 30S subunit.** Out of the three S15-RNA interactions, binding of S15 to 16S rRNA has been the most important and best characterized. S15 interacts with the central domain of 16S rRNA and directs the ordered assembly of S6+S18 dimer, S11 and S21 that bind to this region. S15 and its associated proteins form the “platform” of the 30S subunit. Insights into the nucleotides and amino acid residues involved in RNA-protein interaction have been obtained from the crystal structures of S15 bound to 16S rRNA fragments, either alone or along with S6+S18 in the case of *T. thermophilus*. Further, the binding of S15 to 16S rRNA in *E. coli* has been characterized by a wide range of techniques including footprinting analysis, deletion studies, modification interference, native gel electrophoretic mobility, transient electric birefringence (TEB) and mutational analysis of the RNA.

The 16S rRNA fragment containing the binding site of S15 was isolated by ribonuclease A digestion of S15-16S rRNA complex and it corresponded to part of helix 20, helices 21 and 22. Results from site-directed mutational analysis in the central domain of 16S rRNA and its effect on S15 binding were consistent with previous results from RNase studies. The nucleotides that displayed modified reactivity upon S15 binding were
determined by footprinting analysis using base-specific and hydroxyl radical probing. Base-specific probes footprinted S15 to the purine-rich region in helix 22. S15 also protected nucleotides 724-730 in helix 23. Further, binding of S15 enhanced the reactivity of nucleotides that get protected upon S6/S18 binding. This is consistent with the fact that S15 facilitates the interaction of these proteins to the RNA. Hydroxyl radical footprinting indicated backbone-specific protections in the presence of S15 in the lower part of the 660/750 stem which is in helix 22 suggesting that the binding site of S15 is centered on the three-way helical junction. Lack of hydroxyl radical data in helix 23 and distant constraints further conveyed that protections in this region might be due to secondary effects.

The minimal RNA site that interacts specifically with S15 was identified by construction of a series of deletion mutants followed by native gel electrophoresis mobility shift assays. It was demonstrated that S15 from *B. steraothermophilus* interacts with an RNA oligonucleotide containing nucleotides 585 to 756 (helices 20 to 23) of 16S rRNA with an apparent dissociation constant of 35 nM. Competition binding assay of different deletion mutants of the rRNA fragment further indicated that the major determinant of S15-rRNA interaction is a three-way junction between helices 20, 21 and 22 while helix 23 was dispensable. In addition, it confirmed that helix 22 also contains S15 determinants in the conserved purine-purine rich internal loop. Site-directed mutagenesis and chemical modifications in the three-way helical junction interfered with the binding of S15 to the minimal RNA and these observations were in agreement with earlier findings that two distinct binding regions for the r-protein. Where the three-way junction is indispensable for binding of S15, the G-U/G-C motif contributes only modestly to the interaction. Ethylation modification interference in addition suggested that S15 binding is accompanied
by a conformational change in the RNA leading to orientation of helices 20 and 22 at an acute angle with respect to each other.

The nature of conformational change that is effected by S15 binding was addressed by constructing extended-helical junctions and the relative helical orientation was analyzed using native gel electrophoretic mobility and quantified by TEB. A significant conformational rearrangement was noted in the presence of S15 or Mg$^{2+}$ in the three-way helical junction formed by helices 20, 21 and 22 where the angle between the three helices is nearly equal to $-120^\circ$. Upon binding a conformational change was induced in this region resulting in coaxial stacking of helices 21 and 22 while helix 20 makes an acute angle ($-60^\circ$) with respect to the coaxial stack both in solution and in crystal structures. Since secondary proteins S6 and S18 absolutely require S15 to bind to the central domain of 16S rRNA, this conformational rearrangement resulting from the binding of S15 or Mg$^{2+}$ is likely a crucial organizational step in assembly of this domain. NMR spectroscopy confirmed that the nature of conformations caused by S15 and Mg$^{2+}$ are similar. In addition, equilibrium and kinetic analysis suggested that presence of Mg$^{2+}$ enhances the bimolecular association rate of S15 binding. Thus, in this case S15 might bind to the folded three-way helical junction by tertiary structure capture mechanism.

Crystal structures of S15-rRNA and S15/S6+S18-rRNA further validated the nature of S15 interaction with its binding sites and the conformational change that the r-protein brings about upon interaction. The structures also shed light on the type of interactions between RNA and protein at the two sites highlighting the protein residues that are involved in the interaction for the first time. In the three-way helical junction, protein backbone-RNA backbone and protein side chain-RNA backbone interactions are
predominant. Five of the 17 positive residues of S15 are involved in interacting with this RNA binding site which is in agreement with previous observation that ionic strength only weakly affects the binding of S15 to 16S rRNA. The second site that S15 recognizes is the G-U/G-C motif in the shallow group of helix 22. Side chain amino acids of a loop region in S15 make these contacts. Thus, S15 probably locks down the three-way helical junction and fixes helix 22 in a bent conformation, thus widening the deep-groove in the purine-rich loop. This conformational rearrangement likely opens up binding sites for the other platform proteins, thus facilitating their binding to the RNA leading to the assembly of the platform.

**Assembly of the platform of 30S subunit.** The next step to initiation of folding of the central domain by S15 is the binding of the secondary binders S6 + S18 dimer followed by S11 and then S21. Crystal structure of S15/S6+S18-rRNA brought to light the conformational rearrangement that occurs in the helices 23a and 23b. The assembly mechanism of the central domain proposed on the basis of the crystal structure suggests that in addition to the conformational rearrangement that occurs in helices 20, 21 and 22 as a result of S15 binding, upper three-helix junction formed by helices 22, 23a and 23b is also subjected to a conformational change. This rearrangement results in coaxial stacking of helix 23b and helix 22 with helix 23a folding parallel to helix 22. In addition to facilitating the binding of dimer S6+S18 this rearrangement opens up binding site for S11. The RNP with S15, S6, S18 and S11 interacting with helices 20-23 constitutes the “core” of the central domain and these RNA helices form the minimal binding RNA for the central domain proteins. Not much is known about the tertiary binding central domain protein, S21. There is no S21 in *T. thermophilus* and since all the structural information available is from the
thermophile, the interaction of S21 with 16S rRNA and its contribution towards the assembly of platform are not well understood. Footprinting studies with *E. coli* S21 {powers 1995 RNA} suggest that consistent with its late binding during assembly, this r-protein protects very limited number of nucleotides.

Although S6, S18 and S11 have additional footprints in helices 24-26 of the central domain, these RNA elements are dispensable for the interaction of the platform binders \(^{98}\). This suggested that the RNA which constitutes the central domain can be divided into two classes. The core domain is formed first with S15, S6, S18 and S11 binding to primary elements helices 20-23 and secondary elements (helices 24-26) associate with the preformed core RNP suggesting a hierarchical assembly of the central domain of 30S subunit.

**Dissecting the steps in binding of S15 to 16S rRNA.** Reconstitution of 30S subunit occurs at 42°C *in vitro*. The temperature dependence of reconstitution has been exploited to follow the interaction of S15 with 16S rRNA in a stepwise manner. S15 is bound to 16S rRNA at 0°C and 42°C and the footprints of the r-protein on the rRNA analyzed under both reaction conditions. Hydroxyl radical footprints of S15 \(^{61}\) on 16S rRNA are similar on ice and at elevated temperature indicating that interaction of the protein with the backbone of the RNA occurs with a lower energy of activation. Monitoring base-specific footprints \(^{59}\) of S15 using kethoxal that modifies the guanosines indicate that the bases in the upper part of helix 22 are protected even at the lower temperature. However, footprints in helix 23 show temperature dependence. The guanosines at the 730 region do not show change in reactivity to the chemical probe at 0°C but get protected at 42°C. These results indicate that S15 binds to its two primary binding sites at the three-way helical junction and internal purine-rich loop
in helix 22 in a single step with low activation energy barrier. However, the secondary effects in helix 23 are the result of conformational changes that occur as the next step to the binding of S15. This work is currently ongoing.

**Current study: Conformational change in 16S rRNA surrounding S15.** The crystal structures and footprinting studies have shed light on the nature of RNA-protein contacts and rearrangement of the RNA helices at S15 binding site. However, information is needed on if and how the rRNA- protein interactions change as other proteins bind to the growing RNP during assembly. The conformational changes that occur in the RNA surrounding S15 have been addressed in the following study by employing directed hydroxyl radical probing (discussed below) and S15 as the probe. The study has given insights into specific protein binding events that lead to the folding of the platform and aligning this region with the rest of the domains appropriately to result in functional 30S subunit.

Formation of 30S subunit from its components is accompanied by global compaction resulting from the conformational change induced by the binding of different r-proteins. This work has given insights into the role of different r-proteins that rearrange the RNA elements in the neighborhood of S15 to result in what is found in the complete 30S subunit.

Surprisingly, not all the proteins affecting RNA environment of S15 are platform binding proteins. This suggests that *in vitro* assembly map and domain organization might be over simplication of an otherwise complex process. The structural and footprinting data have been to convert S15 as the probe by substituting cysteine at different locations. These cysteine containing S15 proteins have been used to construct minimal RNPs that represent different stages of assembly. Analysis of RNA environment of S15 in each of these minimal
complexes using directed hydroxyl radical probing gives insights into how the RNA elements rearrange as a result of a protein binding event. The specific protein-dependent RNA conformational changes that reveal subtleties of folding of the central domain and the whole of 16S rRNA upon binding of S15 and other ribosomal proteins during assembly have been uncovered in this study.

**Directed hydroxyl probing: Old technique, New Approach.** Hydroxyl radicals are the smallest probes employed to study nucleic acid-protein interactions. These are generated by Fenton chemistry and attack the ribose moiety. A major advantage of using them as probes is that they cleave the sugar-phosphate backbone of nucleic acids independent of the bases, double-stranded regions or secondary structure adopted by the nucleic acid.

In the past, hydroxyl radicals have been employed in two approaches to address RNA-protein interactions: solution hydroxyl radical probing and directed hydroxyl radical probing. In the case of solution hydroxyl radical probing, hydroxyl radicals are generated with free Fe(II)-EDTA and H₂O₂ in solution where Fe(II)-EDTA is regenerated in the presence of ascorbate. Hydroxyl radicals so produced, cleave naked RNA almost uniformly. In the presence of an RNA binding protein, RNA elements that interact with the protein are protected from getting cleaved. Thus, protection from hydroxyl radical cleavage marks the site of interaction in the RNA. Thus, solution hydroxyl radical footprinting has been used to characterize RNA-protein interactions in 30S subunit for all the proteins as discussed earlier.

In the case of directed hydroxyl radical probing, hydroxyl radicals are generated from unique Fe(II)-tethered sites in the protein and are therefore, "directed". Owing to the limited
range of these radicals, this method has been effective in the past to generate low resolution (10Å-50Å) information about the nucleic acid neighborhood in the vicinity of the molecule of interest. Single cysteine residues are introduced in the protein under study by site-directed mutagenesis (Figure 4(b)) and a Fe(II)-loaded linker, 1-(p-bromoacetamidobenzyl)-EDTA (BABE or Fe(II)-BABE) (Figure 4(a)) is tethered by nucleophilic displacement to the sulfhydryl group of the different cysteines. Structural information and phylogenetic conservation are used as guides for the selection of sites that could accommodate a cysteine residue and the Fe(II)-BABE linker. If there is an inbuilt cysteine in the protein, it is substituted with a non-derivatizable amino acid. The nucleic acid environment of the protein is monitored by producing hydroxyl radicals and following the cleavage generated by the “directed” radicals (Figure 4(b)). The hydroxyl radicals formed from each site cleave only the nucleic acid surrounding the site of production and therefore appearance of cleavage marks the nucleic acid elements in the vicinity of the protein. The advantage of this approach is that RNA-protein interactions can be studied in a macromolecular complex irrespective of the other protein components present in the system.

Directed hydroxyl radical probing has been used extensively in the past to understand nucleic acid environment of proteins in static complexes. For example, it has been used to address the RNA environment of endogenous components such as r-proteins in ribosome and ribosomal subunits. For instance, the location of r-protein S20 was analyzed in 30S subunit and the 16S rRNA elements surrounding S8 in 70S ribosome were mapped using this technique. This approach has been extended to monitor RNA environment of ligands like tRNA. It has been made possible by the tethering of Fe(II)-BABE to in vitro transcripts of tRNA and tRNA analogs via linkage to 5'-phosphorothioate. Before the unraveling of
high resolution crystal structure, this approach led to the understanding of structure of ribosome and interaction with its ligands. This technique has also been used to understand DNA-protein interactions. For instance, RNA polymerase was localized on the promoter sites of DNA by employing directed hydroxyl radical probing.

In the following study, this method has been used to address conformational changes in the 16S rRNA during assembly. Using the recombinant system for in vitro reconstitution, minimal RNPs that represent different stages of assembly were formed with Fe(II)-tethered S15 proteins. The simplest RNP is the binary complex of 16S rRNA and Fe(II)-S15 (Fe(II)-S15/16S rRNA) which represents one of the initial stages of 30S subunit assembly. The other RNPs are formed with addition of different r-proteins. Generation of “directed” hydroxyl radicals leads to cleavage of the 16S rRNA backbone in the vicinity of S15 in different RNPs. Monitoring the difference in the cleavage pattern between the complexes sheds light on the rearrangement of RNA elements in the presence of the respective r-proteins. Thus, conformational changes in the 16S rRNA neighborhood of S15 have been studied using directed hydroxyl radical probing.

Apart from giving insights into the assembly pathway and roles played by r-proteins in this process, the following study demonstrates a novel application of this widely used technique. Directed hydroxyl radical probing can be employed to study rearrangements that occur in nucleic acid-protein complexes during assembly, ligand association or other cellular processes that can be monitored in vitro.
Dissertation Organization

Chapter 1 of this dissertation contains a review of literature. Chapters 2 and 3 have been published in peer-reviewed journals. Chapter 4 provides general summary and future directions. Ongoing work is described in Appendix.

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Figure 1: Some of the first electron micrographs of the ribosome from the laboratory of George palade. (a) free suspension of the ribosomes after solubilization of microsome membrane vesicles. (b) ribosomes attached on the surface of membrane vesicles.
**Figure 2:** Domain organization of 30S subunit. (a) Secondary structure of 16S rRNA with different domains. Head is in blue, platform in red, body in green and penultimate stem in light grey. (b) Tertiary structure of the 16S rRNA with domains in the corresponding colors. (c) *in vitro* assembly map of 30S subunit with proteins binding to the different domains in the respective colors.
Central domain
3' Major domain
3' Minor domain
5' domain

(b) Head
Platform
Body
Penultimate stem

(c) 16S ribosomal RNA
S13
S14
S10
S21
S3
S2
Figure 3: NMR (a) and X-ray structures (b) of ribosomal protein S15. The two main differences between the structures are shown in cyan. 1. In the NMR structure, helix I is positioned against the body, whereas in the crystal structure it is extended away from the molecule. 2. In the NMR structure, loop II is more disordered and helix III is broken up into two helices whereas in the crystal structure, both these regions are highly ordered.
Figure 4: (a) Structure of 1-(p-bromoacetamidobenzyl)-EDTA (BABE), the linker attached to Fe(II) in order to facilitate its tethering to cysteine sites. (b) Scheme for Fe(II)-directed hydroxyl radical probing of RNPs.
Scheme for Fe(II)-directed hydroxyl radical probing of RNPs

1) Generate cysteine containing S15

2) Attach Fe(II)-EDTA via cysteine

3) Reconstitute and Purify RNPs of various complexities

4) Initiate -OH probing

5) Identify sites of cleavage by primer extension
CHAPTER 2: ASSEMBLY OF THE CENTRAL DOMAIN OF THE 30S RIBOSOMAL SUBUNIT ROLES FOR THE PRIMARY BINDING RIBOSOMAL PROTEINS S15 AND S8


Indu Jagannathan and Gloria M. Culver*

Summary

Assembly of the 30S ribosomal subunit occurs in a highly ordered and sequential manner. The ordered addition of ribosomal proteins to the growing ribonucleoprotein particle is initiated by the association of primary binding proteins. These proteins bind specifically and independently to 16S ribosomal RNA (rRNA). Two primary binding proteins, S8 and S15, interact exclusively with the central domain of 16S rRNA. Binding of S15 to the central domain results in a conformational change in the RNA and is followed by the ordered assembly of the S6/S18 dimer, S11 and finally S21 to form the platform of the 30S subunit. In contrast, S8 is not part of this major platform assembly branch. Of the remaining central domain binding proteins, only S21 association is slightly dependent on S8. Thus, although S8 is a primary binding protein that extensively contacts the central domain, its role in assembly of this domain remains unclear. Here we used directed hydroxyl radical probing from four unique positions on S15 to assess organization of the central domain of 16S rRNA as a consequence of S8 association. Hydroxyl radical probing of Fe(II)-S15/16S rRNA and
Fe(II)-S15/S8/16S rRNA ribonucleoprotein particles reveal changes in the 16S rRNA environment of S15 upon addition of S8. These changes occur predominantly in helices 24 and 26 near previously identified S8 binding sites. These S8-dependent conformational changes are consistent with 16S rRNA folding in complete 30S subunits. Thus, while S8 binding is not absolutely required for assembly of the platform, it appears to significantly affect the 16S rRNA environment of S15 by influencing central domain organization.

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Introduction

The major individual domains of 16S ribosomal RNA (rRNA; Figure 1(a)), the 5’, central, and 3’ major, appear as components of distinct structures in 30S ribosomal subunits. This is in marked contrast to the interweaving of 23S rRNA domains observed in 50S subunit structures. Additionally, it has been shown that each 16S rRNA domain, along with appropriate proteins, can assemble in the absence of the other domains to form specific ribonucleoprotein particles (RNPs). While the individual domains can assemble independently, the assembly of a given domain within the context of full 16S rRNA follows a hierarchical and ordered pathway for association of the ribosomal proteins (r-proteins) with 16S rRNA. Within this hierarchical framework, the 30S subunit r-proteins fall into three general assembly classes (Figure 1(b)). The primary binding proteins bind directly and independently to 16S rRNA. The secondary and tertiary binding proteins require the prior association of other proteins before they can assemble. Given this hierarchy, the primary
binding proteins likely play essential roles in orchestrating the assembly of the various domains of 16S rRNA into functional 30S subunits.

The primary binding protein S15 binds directly to and nucleates the folding of the central domain of 16S rRNA concomitant with the association of S6/S18, S11 and S21 to form the platform of the 30S subunit (Figure 1(b)). S15 binds the three-way helical junction formed by helices 20, 21 and 22 and above the purine-rich internal loop in helix 22 (Figure 2). Association of S15 has been shown to induce a conformational change in this three-way helical junction of 16S rRNA. It is likely that this conformational rearrangement of 16S rRNA facilitates the binding of S6/S18, S11 and S21 to the central domain. This is consistent with the lack of protein-protein contacts observed between S15 and S6, S18 or S11 in the 30S subunit. Another primary binding protein that interacts extensively with the central domain of 16S rRNA is r-protein S8 (Figure 2). Footprinting experiments revealed two binding sites within the central domain at/near helices 20, 21, 25 and 26a for S8 (Figure 2). These results revealed novel interactions between S8 and 16S rRNA; previously, it had been demonstrated that helices 20 and 21 contain the minimal binding sequence for S8. Interestingly, one of the two S8 sites is located 5' of the S15 binding site on 16S rRNA and the other is located 3' of the S15 site (Figure 2). Therefore, the S8 binding sites appear to "sandwich" that of S15, suggesting that these proteins are proximal to one another during their association with the central domain. Given the extensive interactions of S8 with the central domain, it is somewhat surprising that S8 does not have a direct role in the binding the platform proteins, S6, S11, and S18 to 16S rRNA and only plays a minor role in S21 association (Figure 1(b)). Indeed, while S8 is located near the platform in the 30S subunit (Figure 3(a)), it is generally considered part of
the body of the 30S subunit (Figure 1(b)). Indeed, it is located near the middle of the back of the body in the 30S subunit structure (Figure 3(a))\(^2;3;17\). This is consistent with a role for S8 in S5 association (Figure 1(b)), although S8 is not the sole protein determinant for S5 binding (Figure 1(b)). These observations might suggest that S8 is not critical for 30S subunit assembly. However, omission of S8 during \textit{in vitro} 30S subunit reconstitution results in a particle that sediments at 24S and is greatly impaired in function\(^20\). Thus, S8 appears to be critical for 30S subunit assembly and might play a significant role in central domain organization that has yet to be revealed.

While biochemical and structural studies have shed light on contacts between the r-proteins S15 and S8 and 16S rRNA\(^2;3;10;11;12;13;14;15;17;21;22\) (Figures 2 and 3(a)), conformational changes that occur during 30S subunit assembly have been harder to study\(^16;23\). Here we have employed directed hydroxyl radical probing to study 30S subunit assembly\(^24\). This method has been effective in studying the rRNA environment of different proteins in ribosomes and ribosomal subunits\(^25;26;27;28;29;30;31;32;33;34\), and thus should be equally useful in studying assembly of these RNPs. In particular protein-dependent 16S rRNA conformational changes that occur during the sequential addition of r-proteins to the maturing 30S subunit could be readily examined. In this study, S15 is employed as a site-directed hydroxyl radical probe to monitor changes in its 16S rRNA neighborhood as a consequence of S8 binding. Four unique cysteine residues have been substituted for other amino acids in the cysteine-less wild type S15 (Figure 3(b)). An Fe(II)-loaded linker, 1-(p-bromoacetamidobenzyl)-EDTA [Fe(II)-BABE]\(^35;36\) was tethered to each of these cysteines, and these proteins were bound to 16S rRNA. Hydroxyl radicals produced from the Fe(II)-modified sites, via Fenton chemistry, cleave the RNA backbone in the vicinity of the probe.
The 16S rRNA environment of S15 was explored in two complexes, Fe(II)-S15/16S rRNA and Fe(II)-S15/S8/16S rRNA. Primer extension analysis revealed significant conformational changes in the 16S rRNA environment of S15 as a consequence of the inclusion of S8, suggesting that S8, along with S15, influences the organization of the central domain.

To understand if the observed changes are relevant to assembly of the complete 30S subunit, we have used the three-dimensional structure of the 30S subunit from *Thermus thermophilus* (*T. thermophilus*)\(^2,4\) as an aid in interpreting our cleavage data (Figure 7). While the high level of conservation between ribosomes from different organisms supports this interpretation, it should be noted that there are some subtle differences in the platform region and in S8 between *Escherichia coli* (*E. coli*) and *T. thermophilus*. The *T. thermophilus* helix 26, which is near one of the two S8 binding sites (Figure 2), is truncated by three base pairs, relative to *E. coli*. Also, *T. thermophilus* S8 has a small insertion relative to the *E. coli* protein. In the crystal structure of the 30S subunit this extended beta hairpin is oriented away from the major 16S rRNA contacts more toward the head of the subunit (Figure 3(a))\(^2,4,17\).

Lastly, *T. thermophilus* 30S subunits lack S21. In spite of these differences, our data are generally in good agreement with the positions of S15 and S8 and contacts between these proteins and 16S rRNA within the 30S subunit\(^2,4,17\) (Figure 7) and suggest that directed hydroxyl radical probing is a valuable tool for understanding the assembly of complex RNP structures.
Results

Construction of Fe(II)-modifiable S15 proteins

Wild-type S15 does not contain any cysteine residues, thus sites for linker attachment were engineered. Structural information and phylogenetic conservation were used to select sites that were generally surface exposed and could accommodate cysteine residues as previously reported. Four positions, 12, 36, 46 and 70, were uniquely substituted for cysteine (Figure 3(b)) and subsequently the proteins were overexpressed and purified. The availability of the cysteines for modification was tested using a thiol-specific fluorescent coumarin reagent (7-diethylamino-3-((4'- (iodoacetyl)-amino)phenyl)-4-methylcoumarin) (DCIA). DCIA derivatization revealed that the cysteine residues of all four proteins were accessible (data not shown), suggesting that each position is available for Fe(II) modification.

Fe(II)-modified S15 proteins bind 16S rRNA

To ensure that neither cysteine-substitution nor Fe(II)-modification altered the function of these proteins, base-specific chemical footprinting experiments were performed. This analysis allows comparison of the previously characterized wild-type S15 footprint on 16S rRNA with the footprints of the modified proteins (Figure 4). As expected wild-type S15 (cysteine-less), untreated (Figure 4, lane 2) or treated with Fe(II)-BABE (mock; Figure 4, lane 3), yields the expected footprint on 16S rRNA. In addition, all four Fe(II)-modified cysteine-substituted S15 proteins reveal footprints on 16S rRNA that are virtually indistinguishable from wild-type (compare Figure 4, lanes 2 and 3 with lanes 4-7). The footprinting results are indistinguishable if the complex is probed pre- (Figure 4) or post-
purification (data not shown). These results and additional studies \(^{(30)}\) (data not shown) indicate that neither cysteine-substitution nor Fe(II)-modification alters the function of these proteins.

**Probing the RNA environment of S15 in Fe(II)-S15/16S rRNA Complexes**

Complexes containing each Fe(II)-modified S15 and 16S rRNA were formed and purified by size exclusion chromatography to remove any free modified protein that might result in spurious hydroxyl radical production. Hydroxyl radicals generated from the Fe(II)-modified sites cleave the RNA backbone proximal to the probe and cleavage sites are identified by primer extension analysis \(^{(40, 41)}\) (Figure 5). The results are mapped on the secondary structure of 16S rRNA (Figure 6(b)) \(^{(42)}\) and on the tertiary structure (Figure 7(b)) of 16S rRNA from the 30S subunit \(^{2; 4}\). As expected, the cleavages are predominantly located near the three-way helical region comprised of helices 20, 21 and 22 where S15 is known to bind \(^{21, 22}\).

Fe(II)-C12-S15 targets nucleotides 746-749 that are in lower portion of helix 22 (Figure 5(c), lane 2). In addition, weaker cleavages are observed from Fe(II) tethered at position 12 at nucleotides 660-663 (Figure 5(b), lane 2). Cleavages from Fe(II)-C36-S15 and Fe(II)-C70-S15 appear in helix 20 at nucleotides 760-763 (Figure 5(c), lanes 3 and 5) and 584-587 (Figure 5(a), lanes 3 and 5). Additionally, Fe(II)-C70-S15 targets the nucleotides 594-596 (Figure 5(a), lane 5) and 650-653 (Figure 5(b), lane 5) in helix 21. All these cleavages are 3' staggered with respect to one another, suggesting minor groove interaction. Fe(II) tethered at position C70 also weakly targets the 770 region at nucleotides 769-772.
(Figure 5(c), lane 5). No distinct cleavages have been observed from C36; all cleavages from Fe(II)-C36-S15 are similar to those from Fe(II)-C70-S15. Also, there is no observed cleavage from Fe(II)-C46-S15 (see Figures 5(a-d), lane 4) in this binary complex. All the cleavage sites around the three-way helical junction are adjacent to actual contacts observed in the tertiary structure of the 30S subunit. Additionally, the general orientation of S15 relative to 16S rRNA based on differential cleavage of sites from each modified position is consistent with the orientation of S15 in the 30S subunit structure (Figure 7).  

**Probing the RNA environment of S15 in S15/S8/16S rRNA RNP**

Hydroxyl radicals were generated from purified Fe(II)-S15/S8/16S rRNA complexes, and cleavage sites were mapped by primer extension analysis (Figures 5, 6(c and d) and 7(e)). Targets cleaved from Fe(II) tethered to different sites on S15 in the Fe(II)-S15/S8/16S rRNA complex are overlapping, yet distinct from those in Fe(II)/S15/16S rRNA complex (Figures 5-7). In addition to cleavage in the three-way helical junction, like those seen in Fe(II)-S15/16S rRNA RNP, additional cleavage sites were observed in helices 24 and 26 (Figures 5-7). Fe(II) linked to C36, C46 and C70 target both the 770 and 810 regions in helix 24 (Figures 5(c and d), lanes 8-10). Nucleotides 808-811 are cleaved from all three of these Fe(II)-tethered sites although the intensity of cleavage is variable. Unlike in the Fe(II)-S15/16S rRNA complex in this S8 containing ternary complex, cleavage was observed from Fe(II)-C46-S15, suggesting that the 16S rRNA environment of this site is altered due to S8 addition. Fe(II)-tethered positions 36 and 70 target nucleotides 769-774 (Figure 5(c), lanes 8 and 10) whereas Fe(II) tethered at position 46 cleaves nucleotides 772-776 (Figure 5(c), lane
9). Additional cleavages from Fe(II)-C12-S15 are observed at nucleotides 836-840 in helix 26 (Figure 5(d), lane 7). The cleavage pattern from Fe(II)-C12-S15 at nucleotides 660-663 (Figure 5(b), lane 7) becomes clearly stronger upon addition of S8. The additional cleavage patterns observed in helices 24 and 26 indicate that these regions are brought in to the vicinity of S15 as a consequence of S8 interaction with 16S rRNA (Figures 6 and 7). These new cleavage sites are consistent with the contacts observed in the 30S subunit structure. Again, the cleavage sites are adjacent to contact sites of S8 and S15.

Additionally, distance measurements between the tethering sites within S15 and the 16S rRNA targets in both complexes are generally consistent with previous tethered hydroxyl radical calibration experiments. These experiments suggest the distance between the probe and target should be within approximately 45Å. All the cleaved residues are within 42Å of the probe attachment site in the 30S subunit structure, except for sites around 770 in helix 24, which are about 50Å away from position 70. These later results fall just outside of the previously determined range for cleavage. One simple explanation is that S11 makes numerous interactions with this helix in the 30S subunit and therefore could play a role in its correct orientation within the 30S subunit. Nevertheless, the extent of agreement is quite remarkable for comparing a complex of 16S rRNA with just one or two proteins bound to the in vivo assembled 30S subunit. These observations suggest important roles for both S15 and S8 in central domain folding and 30S subunit assembly. Additionally, these results reveal that directed hydroxyl radical probing can be used to dissect subtle protein dependent RNA conformational changes during assembly of complex RNPs.
Discussion

Directed hydroxyl probing from various positions on ribosomal protein S15 in complexes with 16S rRNA alone or in combination with S8 has revealed an interesting protein-dependent organization of the central domain. In the S15/16S rRNA complex, Fe(II)-tethered S15 proteins chiefly target the three-way helical junction formed by helices 20, 21 and 22. This is in agreement with this three way helical junction and helix 22 together forming the minimum RNA binding site of S15 and modification interference data regarding important residues for S15 binding. Also, this is generally consistent with the S15 interactions observed within the 30S subunit. In the presence of S8, additional cleavage patterns in helices 24 and 26 were observed indicating that these rRNA elements are closer to or more stably oriented toward S15 when S8 is present (Figures 6 (c and d) and 7 (c-e)). This organization of additional 16S rRNA in the vicinity of S15 upon addition of S8 is consistent with global compaction thought to occur during 30S subunit assembly and with the structure of 30S subunit. These results reveal some of the intricacies involved in the folding of the central domain and may have significant implications for binding of additional proteins to the central domain. Ribosomal proteins S6, S18 and S11 depend on S15 for their association with 16S rRNA (see Figure 1(b)). These proteins have footprints in helices 20-24 and 26, and these data are overall consistent with contacts within the 30S subunit. Previous structural work revealed that helices 20-23 are sufficient for binding the central domain proteins S6, S8, S11, S15 and S18 and allowed speculation that helices 24-26 contain secondary binding sites for some of these proteins. Our data indicates that addition of S8 induces a conformational change in the central domain bringing helices 24 and 26 proximal to S15. It is possible that this conformational change may facilitate the binding of S6, S18
and S11; however, this remains to be seen. Helices 22, 23a and 26 are closely packed in 30S subunit, and this packing appears to be stabilized by direct contact of S18. These results are consistent with a crucial role for S8 in organizing some helices in the central domain of 16S rRNA and facilitating formation of the platform of the 30S ribosomal subunit.

A critical role for S8 in central domain folding is further supported by analysis of the 16S rRNA binding sites for S8: the minimum binding site for S8 resides in helix 21, but S8 also footprints helix 25. It has previously been proposed that S8 might interact with its two distinct binding sites at different stages of assembly. This bipartite binding theory for S8 is supported by a co-transcriptional or a 5' to 3' model of 30S subunit assembly. As 16S rRNA is transcribed, helix 21 will be produced first and thus would present an initial binding site for S8. Subsequently, as the rest of the central domain is transcribed, helix 25 would form and S8 could bind this secondary site. Additionally, in 30S subunits two separate domains of S8 are responsible for interacting with these binding sites. It is intriguing that the S15 binding site is located between the two S8 elements; thus S15 could bind subsequent to S8 interaction with helix 21 but prior to S8 interaction with helix 25. This sort of binding sequence would reveal another level of the hierarchical 30S subunit assembly process. Indeed, some critical folding of the central domain of 16S rRNA mediated by a combination of S15 and S8 may not be fully revealed until co-transcriptional 30S subunit assembly is studied.

Given that the individual domains of the 30S subunit can form independently, it follows that the orientation of these domains relative to one another is an essential step in functional 30S subunit formation. S8 may play a critical role in domain orientation. Helix 21 contains the minimal binding site for S8, and this helix is found crossing the back
(solvent surface) of the body of the 30S subunit. Thus this portion of the central domain and S8 are closely aligned with the 5' domain of 16S rRNA. The binding of S8 to helix 21 and helix 25 may not only aid platform formation but may also position the platform relative to the body of the emerging 30S subunit. Additionally, S8 is important for S5 binding during assembly (Figure 1(b)) and makes contacts with S5 in the 30S subunit. S5 bridges the body and the head of the 30S subunit and maybe critical for the orientation of these two domains. Therefore, both directly and indirectly S8 could be involved in aligning the three major domains of the 30S subunit. This hypothesis could explain the dramatic effects of S8 omission in vitro reconstitution studies that are not readily explained by simple assembly dependencies (Figure 1(b)). A critical role for S8 in orienting the major domain structures of the 30S subunit would explain its importance to 30S subunit function and underscore the role for S8 in central domain organization that was demonstrated in this work.

Materials and Methods

Mutagenesis, expression and purification of S15

The gene-encoding ribosomal protein S15 was previously cloned from E. coli MRE600 genomic DNA into pET24b vector (Novagen). The cysteine residues were introduced at four non-conserved positions (12, 36, 46 and 70) by site directed mutagenesis and confirmed by sequence analysis. Each mutant S15 protein was expressed in E. coli BL21 and purified as previously described for wild type protein.
Derivatization of S15 proteins

The accessibility of each introduced cysteine residue for derivatization was assessed by reaction with the fluorescent reagent 7-diethylamino-3-((4'-(iodoacetyl)amino)phenyl)-4-methylcoumarin (DCIA; Molecular Probes). Wild-type S15 was assayed in parallel with the cysteine-containing S15 mutant proteins to assess non-specific derivatization at positions other than cysteines. Derivatization of cysteine-containing S15 proteins by DCIA and Fe(II)-BABE was done as previously described.

Formation of Fe(II)-S15/16S rRNA complexes

40pmoles of natural 16S rRNA (isolated from natural 30S subunits as described) was pre-incubated at 42°C for 15 minutes in buffer A (20mM K+-Hepes (pH 7.6), 20mM MgCl₂). 240pmoles of Fe(II)-S15 were added and incubated at 42°C for 60 minutes. The KCl concentration was adjusted to 330mM in each of the reactions in a final volume of 100μL. The complexes were stabilized by placing the reactions on ice for 15 minutes. The complexes were used directly in footprinting experiments or purified from Fe(II)-S15 for directed hydroxyl radical probing (see below).

Formation of Fe(II)-S15/S8/16S rRNA complexes

The Fe(II)-S15/S8/16S rRNA complexes were formed in two different ways and both methods yielded identical results for both footprinting of S15 and S8 (data not shown) and the directed hydroxyl radical cleavage experiments. In one case, 240pmoles of S8 was added
to the purified Fe(II)-S15/16S rRNA complexes (formed as described above), the KCl concentration was adjusted to 330mM final volume of 100μL and the reactions were incubated at 42°C for 60 minutes. The complexes were stabilized by placing the reactions on ice for 15 minutes. The complexes were used directly in footprinting experiments or purified from Fe(II)-S15 for directed hydroxyl radical probing (see below). In the second case, 240pmoles of Fe(II)-S15 and 240pmoles of S8 were added to 40pmoles of heat activated natural 16S rRNA (see above) and incubated at 42°C for 60 minutes. The KCl concentration was adjusted to 330mM in each of the reactions in a final volume of 100μL. The complexes were stabilized by placing the reactions on ice for 15 minutes. The complexes were used directly in footprinting experiments or purified from Fe(II)-S15 for directed hydroxyl radical probing (see below).

**Footprinting S15 on 16S rRNA**

Footprinting of Fe(II)-S15 proteins on 16S rRNA with the base-specific probe, kethoxal which modifies N-1 of G was done as described by Merryman and Noller 46.

**Purification of complexes from Fe(II)-S15**

Prior to tethered hydroxyl radical probing, the complexes made with Fe(II)-S15 proteins were purified to remove any unbound protein. This was done as described by Culver and Noller 41. The recovered samples were immediately subjected to hydroxyl radical probing.
Directed hydroxyl radical probing

Directed hydroxyl radical probing of 16S rRNA from Fe(II)-S15 and subsequent primer extension analysis was done as described by Culver and Noller \(^{41}\).

Measurements of Distances between Fe(II)-tethered sites and targets within 16S rRNA

The program MOLMOL \(^{47}\) was used for calculating distances between the alpha carbon of the natural amino acid at the tethering site within S15 and the phosphate atom in 16S rRNA.

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**Figure 1.** Domain organization of the 30S subunit. (a) The secondary structure of 16S rRNA from *E. coli*\(^{42}\). The major domains are colored: 5’ domain, gray; central domain, pink; 3’ major domain, green; 3’ minor domain, black. (b) *In vitro* assembly map of 30S ribosomal subunits. The primary binding proteins are shown in black and the secondary and tertiary binding proteins are shown in blue. The three structural domains are colored to match the corresponding major 16S rRNA element shown in panel (a). The general domain structural names are also given in corresponding colors. S6 and S8 are shown in a dashed line box because they bind as a heterodimer. This assembly map is slightly simplified and modified\(^{48}\) from the original map\(^{49,50}\).
(a)

central domain

5' domain

3' minor domain

(b)

16S ribosomal RNA

platform

S15
S17
S16
S12
S18
S19
S13
S10
S3
S2
Figure 2. Combined base-specific and hydroxyl radical footprinting data for S15 (circle) and S8 (diamond)\textsuperscript{12,13} shown on the central domain of 16S rRNA. Major RNA helices are numbered in accordance with earlier work\textsuperscript{51}.
**Figure 3.** Structural components of the 30S ribosomal subunit. (a) Two views of the three-dimensional structure of the 30S ribosomal subunit from *T. thermophilus*[^2][^4]. S15 is shown in black, S8 is shown in orange, all other proteins are shown in gray and 16S rRNA is shown in cyan. (b) Structure of S15 (taken from Fig. 3(a)) with sites of cysteine substitutions indicated. Position 12 is shown in blue, 36 in magenta, 46 in yellow and 70 in green.
**Figure 4.** Protection of nucleotides in 16S rRNA by Fe(II)-modified S15 proteins from modification with kethoxal. A, G: sequencing lanes; K: unmodified 16S rRNA. RNA in all other lanes was modified with kethoxal after complex formation with 16S: no protein; wt: wild-type S15, wt mock: Fe(II)-BABE treated wild type S15 protein; C12: Fe(II)-C12-S15; C36: Fe(II)-C36-S15; C46: Fe(II)-C46-S15; C70: Fe(II)-C70-S15. Primer extension analysis of the 730 region extended from primer 795 is shown. Arrows indicate sites of protection or enhancement.
Figure 5. Directed hydroxyl radical cleavage of 16S rRNA from Fe(II)-S15 in Fe(II)-
S15/16S rRNA (left) and Fe(II)-S15/S8/16S rRNA (right) complexes analyzed by primer
extension. A, G: sequencing lanes. All other lanes are from Fe(II)-S15/16S rRNA or Fe(II)-
S15/S8/16S rRNA complexes containing wt: Fe(II)-BABE treated wild type S15; C12:
Fe(II)-C12-S15; C36: Fe(II)-C36-S15; C46: Fe(II)-C46-S15; C70: Fe(II)-C70-S15. Primers
used for analysis were 795 and 939. Bars at the right indicate regions of 16S rRNA cleavage.
Figure 6. Hydroxyl radical cleavage sites shown on the secondary structure of 16S rRNA. (a) Secondary structure of 16S rRNA with the central domain highlighted. This highlighted domain is expanded in panels (b), (c) and (d). (b) represents sites cleaved from Fe(II)-S15 in Fe(II)-S15/16S rRNA; (c) represents new sites cleaved from Fe(II)-S15 in Fe(II)-S15/S8/16S rRNA and (d) summarizes sites cleaved from Fe(II)-S15 in Fe(II)-S15/S8/16S rRNA. The target nucleotides are marked by colored circles corresponding to cysteine substituted sites on S15 in Fig. 3 (b). Concentric rings of color represent cleavage of a nucleotide from Fe(II) tethered to more than one position.
Figure 7. Hydroxyl radical cleavages mapped on to the three-dimensional structure of *T. thermophilus* 30S ribosomal subunit. (a) Three-dimensional structure of *T. thermophilus* 30S ribosomal subunit with the platform boxed, 16S rRNA is shown in cyan, S15 is shown in black with cysteine attachment sites colored as in Fig. 3(b) and S8 is shown in orange. The boxed region is expanded in the panels (b), (c), (d) and (e) and some helix numbers are given. (b) represents sites cleaved from Fe(II)-S15 in Fe(II)-S15/16S rRNA complex, (c) represents new sites cleaved from Fe(II)-S15 in Fe(II)-S15/S8/16S rRNA, (d) summarizes sites cleaved from Fe(II)-S15 in Fe(II)-S15/S8/16S rRNA and (e) same as (d) with 180° rotation. The position of phosphate backbone that is cleaved is marked by colored circles corresponding to the site. Concentric rings of color represent cleavage on a nucleotide from Fe(II) tethered to more than one position.
CHAPTER 3: RIBOSOMAL PROTEIN-DEPENDENT ORIENTATION OF THE 16S rRNA ENVIRONMENT OF S15

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Summary

Ribosomal protein S15 binds specifically to the central domain of 16S ribosomal RNA (16S rRNA) and directs the assembly of four additional proteins to this domain. The central domain of 16S rRNA along with these five proteins form the platform of the 30S subunit. Previously, directed hydroxyl radical probing from Fe(II)-S15 in small ribonucleoprotein complexes was used to study assembly of the central domain of 16S rRNA. Here, this same approach was used to understand the 16S rRNA environment of Fe(II)-S15 in 30S subunits and to determine the ribosomal proteins that are involved forming the mature S15-16S rRNA environment. We have identified additional sites of Fe(II)-S15-directed cleavage in 30S subunits compared to the binary complex of Fe(II)-S15/16S rRNA. Along with novel targets in the central domain, sites within the 5' and 3' minor domains are also cleaved. This suggests that during the course of 30S subunit assembly these elements are positioned in the vicinity of S15. Besides the previously determined role for S8, roles for S5, S6+S18, and S16 in altering the 16S rRNA environment of S15 were established. These studies reveal that ribosomal proteins can alter the assembly of regions of the 30S subunit...
from a considerable distance and influence the overall conformation of this ribonucleoprotein particle.

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Introduction

Assembly of the *Escherichia coli* (*E. coli*) 30S ribosomal subunit requires the hierarchical addition of a unique set of small subunit ribosomal proteins (r-proteins) to 16S ribosomal RNA (rRNA). 16S rRNA is composed of 1542 nucleotides, which are distributed in four distinct secondary structural domains, the 5', central, and 3' major and minor domains. A series of elegant single r-protein addition and omission experiments\(^1\):\(^2\) resulted in the production of a 30S subunit *in vitro* assembly map which reveals the requirements for and order of r-proteins binding to 16S rRNA (Figure 1). Small subunit ribosomal proteins can be divided into three major groups based on their 16S rRNA or ribonucleoprotein particle (RNP) association properties. R-proteins that associate independently and specifically with 16S rRNA are termed primary (1°) binding proteins. The secondary (2°) binding proteins require that a primary binding protein be associated before they bind. The r-proteins that minimally require prior addition of both primary and secondary binding proteins are referred to as tertiary (3°) binding proteins. R-proteins from these different categories are interrelated and form networks that are revealed in the *in vitro* assembly map as distinct assembly branches (see Figure 1). The proteins associated with the assembly branches often interact
with a specific 16S rRNA domain to form discrete, individual structural elements of the 30S subunit.

The primary binding protein S15 has been implicated in orchestrating events during 30S subunit assembly and is the foundation for an assembly branch (Figure 1). S15 binds to the central domain of 16S rRNA at the three way helical junction formed by helices 20-22 and above the purine-rich internal loop in helix 22 (Figure 2(a)). A conformational change in this region occurs upon S15 binding, and S15 association is required for the binding of four other r-proteins: S6, S11, S18 and S21. The central domain of 16S rRNA along with the proteins found in the S15 assembly branch (Figure 1) form the platform of the 30S subunit, which interacts with tRNA, the 50S subunit and translational factors during the course of protein synthesis. A challenge in understanding 30S subunit assembly involves identifying changes that occur during the process which ultimately result in a mature, functional 30S subunit. The three-dimensional arrangement of elements within 30S subunits has been identified in crystallographic structures and from this data, the 16S rRNA environments of individual proteins have been revealed. However these structures cannot describe the assembly process that results in the formation of specific environments.

Recently, we have demonstrated that directed hydroxyl radical probing from S15 could be used to monitor protein-dependent changes in the 16S rRNA neighborhood of S15 using minimal RNPs. Directed hydroxyl radical probing from unique positions in S15 (Figure 2c) was performed in two complexes: Fe(II)-S15/16S rRNA and Fe(II)-S15/S8/16S rRNA. These experiments revealed that the 16S rRNA environment was altered in an S8-dependent manner thereby suggesting a crucial role for S8 in organizing the central domain of 16S rRNA and subsequently forming the platform of the 30S subunit.
Here, additional changes in the 16S rRNA elements surrounding S15 have been investigated. Significant cleavages in helices 11, 22, 24, 26, 27 and 44 were observed from Fe(II)-S15 in 30S subunits that were not present in Fe(II)-S15/16S rRNA or Fe(II)-S15/S8/16S rRNA complexes, indicating that these elements are proximal to S15 in the 30S subunit. The cleavage sites in helices 24 and 26 of the 30S subunit are similar to yet distinct from those we reported for the ternary complex, Fe(II)-S15/S8/16S rRNA. Specific r-proteins responsible for orchestrating changes within the S15 environment were identified. Interestingly, many of the proteins that influence the RNA neighborhood of S15 are found outside of the platform and are not part of the S15 assembly branch. Important roles for the r-proteins (S5, S6+S18, S16) in organization of 16S rRNA in the vicinity of S15 during 30S subunit formation have been revealed and suggest that ribosome assembly can be mediated by r-proteins that act between domains and at a significant distance.

Results

Reconstitution of Fe(II)-S15 containing 30S subunits

Previously, four S15 proteins each containing a unique cysteine residue at positions 12, 36, 46 or 70 were constructed (Figure 2). Initially it appeared that C36 was refractory to derivation, yet in more recent studies modification of C36 was observed. It was demonstrated that modification of these cysteine-substituted proteins with the Fe(II)-loader linker, 1-(p-bromoacetamidobenzyl)-EDTA (Fe(II)-BABE) does not interfere with the ability of the proteins to bind to 16S rRNA. Here, the same Fe(II)-modified S15 proteins were used to reconstitute 30S subunits in vitro. Reconstitutions containing natural
16S rRNA, a complete recombinant protein system \(^{21}\) including either wild-type S15 (cysteine-less, but mock treated with Fe(II)-EDTA linker) or one of the four Fe(II)-S15 proteins, produce particles that co-sediment with 30S subunits (Figure 3).

**Reconstituted 30S subunits containing Fe(II)-S15 are active in tRNA binding**

While it was previously demonstrated that the Fe(II)-deriviatization of any of the four mutant S15 proteins did not alter 16S rRNA binding \(^{17}\), it is important to determine if inclusion of these proteins in 30S subunits interfered with tRNA binding, i.e. 30S subunit function. No significant effect on the tRNA binding capabilities of 30S subunits containing Fe(II)-S15 was observed (Table 1). These results along with previous work \(^{17}\) suggest that modification of S15 at these four sites does not perturb interactions that are important for 16S rRNA or tRNA binding.

**Probing the RNA environment of S15 in 30S subunits**

Hydroxyl radical probing was performed using sucrose gradient purified Fe(II)-S15-30S subunits \(^{22}\). Cleavage sites in 16S rRNA were identified by primer extension analysis \(^{23}\) (Figure 4) and are mapped on the secondary structure of 16S rRNA \(^{24}\) (Figure 5 and 7c and d) and on the tertiary structure of 16S rRNA from the 30S subunit of *Thermus thermophilus* (*T. thermophilus*) \(^{16}\) (Figure 6 and 7a and b). Cleavage of the three-way helical junction of helices 20, 21 and 22 is observed in Fe(II)-S15-30S subunits as in the Fe(II)-S15/16S rRNA complexes \(^{17}\). In addition, helices 11, 24, 26, 27 and 44 contain targets cleaved in Fe(II)-S15-30S subunits. More subtle cleavage of elements in helices 22 and 24 are also observed in Fe(II)-S15-30S subunits. Some of the additional cleavages in helices 24 and 26 are similar to
those observed in the ternary complex, Fe(II)-S15/S8/16S rRNA; the others appear to be novel sites of cleavage for Fe(II)-S15.

Within the 5' domain of 16S rRNA, Fe(II)-S15-30S subunit cleavage sites are localized to helix 11 (Figure 5). Cleavages are observed at nucleotides 243-247 and 274-280 from Fe(II)-C70-S15 (Figure 4(a), lane 5). No other nucleotides in the 5' domain appear to be targets for Fe(II)-C70-S15, and none of the other Fe(II)-derivatized S15 proteins target the 5' domain.

Many central domain cleavage sites are similar to those previously observed in the Fe(II)-S15/16S rRNA complexes. Nucleotides 660-663 (Figure 4(c), lane 2) and 746-749 (Figure 4(d), lane 2) in helix 22 are targeted by Fe(II)-C12-S15. Cleavages from Fe(II)-C36-S15 and Fe(II)-C70-S15 appear at nucleotides 584-587 (Figure 4(b), lanes 3 and 5) and 760-763 (Figure 4(d), lanes 3 and 5). Fe(II)-C70-S15 also targets nucleotides 594-596 (Figure 4(b), lane 5) and 648-651 (Figure 4(c), lane 5) in helix 21. All of the above cleavages were also observed in the binary Fe(II)-S15/16S rRNA complexes. Some of the observed targets in Fe(II)-S15-30S subunits are similar to those in these ternary complex of Fe(II)-S15/S8/16S rRNA. Nucleotides 769-774 in helix 24 are cleaved from Fe(II)-C36-S15 and Fe(II)-C70-S15 (Figure 4(d), lanes 3 and 5) as are nucleotides 772-777 from Fe(II)-C46-S15 (Figure 4(d), lane 4) in both of these types of complexes.

There are sites within the central domain which are targets for Fe(II)-S15-30S subunits that were not previously observed. Cleavages from Fe(II)-C46-S15 in helix 22 at nucleotides 669-671 (Figure 4(c), lane 4) and in helix 24 at nucleotides 775-777 (Figure 4(d), lane 4) are observed in the 30S subunits. Fe(II)-tethered to positions 36, 46 and 70 target nucleotides 769-774 (Figure 4(d), lanes 3-5) and nucleotides 808-811 in helix 24 (Figure
4(e), lanes 3-5) with the sites most strongly cleaved from Fe(II)-C46-S15. Fe(II)-C12-S15 also targets nucleotides 808-811 (Figure 4(e), lane 2) in addition to nucleotides 836-843 in helix 26 (Figure 4(e), lane 2). Nucleotides 896-899 (Figure 4(f), lane 5) in helix 27 are targeted from Fe(II)-C70-S15. In this same region, nucleotides 898 and 899, are also cleaved by Fe(II)-C46-S15 (Figure 4(f), lane 4) in Fe(II)-S15-30S subunits.

Regions of the penultimate stem, helix 44, are targeted by Fe(II)-S15-30S subunits, while these cleavages were not observed in the Fe(II)-S15 or Fe(II)-S15/S8 16S rRNA complexes. Fe(II)-S15-C70 weakly targets nucleotides 1416-1418 and 1426-1432 in helix 44 (Figure 4(g), lane 5). These sites are appropriately spaced from one another to fall on the same face of the helix.

The majority of the Fe(II)-S15-30S subunit cleavage results are generally consistent with the location of the tethering positions on S15 and the position of the target sites within the 30S subunit. Calibration experiments indicate that targets should be within approximately 45 Å of the probing sites. Distance measurements reveal that all of our target sites are within this range from the probing site except for the cleavages in the penultimate stem by Fe(II)-S15-C70. These 16S rRNA sites are approximately 50-69 Å from position 70 on S15. The cleavage events in helix 44 are some of the weakest observed in the 30S subunit, and thus should be most remotely positioned relative to S15 as is consistent with these results. Comparison of different structures of 30S subunits suggests that the penultimate stem can occupy slightly different conformations depending on ligand-bound state and subunit association of the 30S subunit. Additionally, the sites of S15 cleavage are interspersed with subunit association sites on the 30S subunit. Thus, it is possible that in the in vitro reconstituted Fe(II)-S15-30S subunit which has seen no ligands, helix 44 occupies a slightly
altered conformation than in 30S subunits used in structural studies. It is also possible that slight differences in distances may result from comparison of our *E. coli* data with structures of *T. thermophilus* 30S subunits \(^{16}\).

**Probing Fe(II)-S15 containing RNPs**

The appearance of Fe(II)-S15 directed cleavages of 30S subunits in helices 11, 24, 26, 27 and 44 that are not found in binary Fe(II)-S15/16S rRNA complexes suggests that these regions undergo conformational rearrangement during 30S ribosomal subunit assembly. These changes likely result from protein-dependent RNA conformational changes.

Information, taken from the assembly map (Figure 1) as well as 30S subunit structures \(^{12}\), was used to identify proteins that might influence the 16S rRNA environment of S15 in this manner. Likely candidate r-proteins were added to complexes containing 16S rRNA and Fe(II)-S15 which were then purified prior to hydroxyl radical probing to remove free Fe(II)-S15 which could result in spurious cleavage.

**Targets attributed to S8**

Previously, it was shown that addition of S8 to the Fe(II)-S15/16S rRNA complex brought about conformational changes in helices 24 and 26 \(^{17}\). The majority of the cleavages seen in Fe(II)-S15-30S in these helices can be attributed to S8. However, there are subtle differences between the cleavage patterns of the ternary complex of Fe(II)-S15/S8/16S rRNA and Fe(II)-S15-30S subunits. In 30S subunits, Fe(II) tethered to all four positions of S15 target nucleotides 808-811 with cleavage from Fe(II)-C46-S15 (Figure 4(e), lanes 3-5) being significantly stronger than the others. Whereas, in Fe(II)-S15/S8/16S rRNA RNP nucleotides
808-811 are targeted by Fe(II) from positions C36, C46 and C70 with equal intensity and
Fe(II)-C12-S15 does not target these sites. Also, slight differences are found in the sites
cleaved in the loop of helix 26. The above differences indicate that the relative positions of
helices 24 and 26 and r-protein S15 are slightly different in 30S subunits than in Fe(II)-
S15/S8/16S rRNA complexes. The observed differences are consistent with the location of
these elements in the 30S subunit indicating that the positioning of these 16S rRNA
elements is refined during 30S subunit assembly.

Role of the platform proteins: S6+S18, S11 and S21

Four r-proteins, S6, S18, S11 and S21, are dependent on S15 for their association
with 16S rRNA during 30S subunit assembly (Figure 1). These proteins all bind the central
domain of 16S rRNA and once assembled form the platform of the 30S subunit. Thus, these
four platform proteins might play a reciprocal role in assembly by altering the 16S rRNA
neighborhood of S15. The RNA environment of S15 in each of the complexes: Fe(II)-
S15/S6+S18/16S rRNA (S6 and S18 bind as a heterodimer), Fe(II)-S15/S6+S18/S11/16S
rRNA and Fe(II)-S15/S6+S18/S11/S21/16S rRNA (see Figure 1) was studied. Compared to
Fe(II)-S15/16S rRNA complexes, additional cleavages were observed in the presence of S6
and S18 at nucleotides 669-671 in helix 22 (Figure 4(c), lane 9) and nucleotides 769-772 in
helix 24 from Fe(II)-C46-S15 (Figure 4(d), lane 9). These cleavages are also observed in
Fe(II)-S15-30S subunits, although the relative cleavage intensities differ slightly between the
RNPs and 30S subunits. No additional targets are cleaved in the presence of S11 or S21 (data
not shown). This lack of additional cleavage upon addition of S21 was also observed using
RNPs containing 16S rRNA, Fe(II)-S15, the remaining primary binding proteins, the
secondary binding proteins and S21 (data not shown). This suggests that the platform proteins S6 and S18, which bind as a heterodimer, orchestrate a slight change in the 16S rRNA environment of S15. However, the platform proteins alone are not sufficient for full organization of the mature 16S rRNA context of S15.

Role of other r-proteins in organizing the RNA environment of S15

Although it has been demonstrated that the three major domains of 16S rRNA can assemble independently, synergy between assembly of the domains and relative orientation of the domains is likely. Therefore, r-proteins that footprint regions (see 9) or make contacts with the RNA in the 5', central or 3' minor domains near the Fe(II)-S15 cleavage sites might play a role in rearranging the RNA environment of S15. In order to evaluate the potential roles of these proteins, RNPs with Fe(II)-modified S15 and 16S rRNA in the presence of different r-proteins were formed and systematically analyzed for conformational changes as detected by 16S rRNA cleavage.

i) Primary binding proteins

Three primary binding proteins, (excluding S8 which has already been addressed) S4, S17 and S20, are likely candidates for influencing the 16S rRNA environment of S15. These proteins interact with multiple helices in different domains of 16S rRNA that are proximal to the Fe(II)-S15 target sites (see 9, 12). In order to analyze the roles of the primary binding proteins in determining the 16S rRNA environment of S15, RNPs containing Fe(II)-S15 and the remainder of the primary binding proteins were probed. In these complexes, no cleavage was observed in helices 11, 27 or 44, suggesting that the primary binding proteins
alone are not sufficient for reorganization of these domains. Cleavages observed in the Fe(II)-S15/1°/16S rRNA complexes were similar to those seen in the ternary complex of Fe(II)-S15/S8/16S rRNA, consistent with the presence of the primary binding protein S8 in both complexes. Footprinting revealed that the primary binding proteins were associated with the RNP under the probing conditions (data not shown).

ii) Secondary binding proteins

Among the secondary binding proteins, roles for S6 and S18 in organizing the RNA elements around S15 have already been discussed. To determine if other 2° binding proteins also participate in the organization of the 16S rRNA environment of S15, RNPs containing Fe(II)-S15/1°/2°/16S rRNA were reconstituted and probed. Interestingly, cleavages in helices 11 and 27 were observed in these complexes although some of the cleavages were weaker than their counterparts in 30S subunits. This indicates that secondary binding proteins, in addition to S6+S18 are responsible for bringing these RNA elements proximal to S15. Analyzing the footprints of this group of proteins with 16S rRNA revealed a set of likely candidates, S16, S5 and S12.

Ribosomal protein S16

Both footprinting and structural data suggest that S16 might influence the alignment of the 5', central and 3' minor domains. Since none of the primary binding proteins, except S8, has an influence on the 16S rRNA environment of S15 and since S16 is dependent on two 1° binding proteins, complexes containing Fe(II)-S15, all the remaining primary binding proteins, S16 and 16S rRNA were formed and subjected to directed
hydroxyl radical probing. Cleavage in helix 11 in the 250/280 region from Fe(II)-C70-S15 was observed in the Fe(II)-S15/1°/S16/16S rRNA complexes. This pattern is similar to that observed in Fe(II)-30S subunits (Figure 4(a), lanes 5 and 10), but is lacking in Fe(II)-S15/1°/16S rRNA complex (data not shown), suggesting that positioning of helix 11 proximal to S15 is an S16-dependent event. In some instances, it was possible to observe very weak cleavage, of an even lesser intensity than that observed in Fe(II)-S15-30S subunits, in helix 44 in the Fe(II)-S15/1°/S16/16S rRNA complexes. However, given the low level of cleavage at these sites, it is not possible to definitively attribute the positioning of helix 44 to S16. All the other cleavages in these complexes were previously attributed to S8.

Ribosomal proteins S5 and S12

R-protein S5 has base-specific footprints in the multibranch loop at the junction of the three major domains. The binding of S5 to the pre-30S subunit is dependent on the prior association of S4, S16 and S8 with 16S rRNA (see Figure 1). In the 30S subunit, S5 contacts both the 5' and central domains of 16S rRNA, as does r-protein S12. S12 is dependent on the prior assembly of S5 and S17 for its association with the pre-30S subunit. Thus, S5 and S12 were tested as potential candidates for influencing the 16S rRNA environment of S15. Toward this end, RNPs with S5 (Fe(II)-S15/1°/S16/S5/16S rRNA) or S5 and S12 (Fe(II)-S15/1°/S16/S5/S12/16S rRNA) were reconstituted. Cleavage in helix 27 was detected in both S5 containing RNPs (Figure 4(f), lanes 9, 10, 14 and 15). However, the intensity of the cleavage at these targets differs between the more minimal particles and the Fe(II)-S15-30S subunits. This suggests that r-protein S5 plays a role in orienting helix 27, which is proximal to the convergence of all the secondary structural domains of 16S rRNA, and S15 nearer to
one another. Moreover, since the strength of cleavage in helix 27 does not increase in the presence of S12 in the Fe(II)-S15/1°/S16/S5/S12/16S rRNA RNP, S12 might not play a significant role in orienting this helix. No other target sites were influenced by the presence of S5, S12 or both proteins.

These studies have revealed protein-dependent 16S rRNA conformational changes that are critical for forming the mature environment of S15. Interestingly, proteins responsible for all cleavages have been identified, except for those cleavage sites in helix 44, the penultimate stem of 16S rRNA. However, it does appear that there are subtle changes between cleavages in the RNPs and those observed in the full 30S subunits. Our data suggest that information is communicated between different domains throughout the assembling subunit. This is likely a common theme in assembly of nucleic acid containing macromolecular complexes.

**Discussion**

Directed hydroxyl radical probing analysis of the 16S rRNA environment of S15 in 30S subunits was performed, and these cleavage patterns were compared with those previously obtained in minimal Fe(II)-S15 complexes. This comparison suggests that conformational changes occur which alter the environment of S15 during 30S subunit assembly. In the Fe(II)-S15/16S rRNA complex, most targets were located in the three-way helical junction formed by helices 20, 21 and 22. In Fe(II)-S15-30S subunits, along with elements in this three-way helical junction, helices 11, 24, 26, 27 and 44 were also targets for cleavage by hydroxyl radicals produced at different Fe(II)-tethered sites on S15. Weak target sites were also observed in helices 22 and 24 in Fe(II)-S15-30S subunits. Thus, the only
domain of 16S rRNA that is not cleaved in Fe(II)-S15-30S subunits is the 3' major domain. Our data generally are in good agreement with the location of these elements in 30S subunits \(^{12;16}\). Previously, it was shown that S8 plays a role in altering the 16S rRNA neighborhood of S15. These results are consistent with our 30S subunit probing data and with the structure of the 30S subunit \(^{12}\). In order to determine which r-proteins account for the other cleavages in Fe(II)-S15-30S subunits, RNPs containing Fe(II)-S15 and different r-proteins were systematically constructed and subjected to directed hydroxyl radical probing. Roles for r-proteins S5, S6+S18 and S16 were determined, indicating specific proteins are involved in protein-dependent 16S rRNA conformational changes during the assembly of the 30S subunit.

Four r-proteins, S6+S18, S11 and S21, require prior association of S15 with 16S rRNA before they can bind to the pre-30S RNP (Figure 1). Of these proteins, S6 and S18 are directly dependent on S15 for binding as a heterodimer. In the presence of S6 and S18, Fe(II) tethered to C46 of S15 cleaves the upper part of helix 22. These results are in good agreement with the 30S subunit structure, which reveals that both S6 and S18 make contacts with the upper portion of helix 22 \(^{12;16}\). In addition, there are subtle changes in the cleavage pattern from Fe(II)-C46-S15 in helix 24 in Fe(II)-S15/S6+S18/16S rRNA. This cleavage pattern is similar to, yet distinct from, one observed in the Fe(II)-S15/S8/16S rRNA complex \(^{17}\). Helix 24 is oriented toward the top of the platform and is positioned between S15 and S6+S18, while S8 is located slightly to one side of this helix in the 30S subunit \(^{12;16}\). Since these three proteins can alter the location of elements in helix 22, it may be that this helix is particularly sensitive to changes in the organization of the central domain. The correct positioning of helix 24 may be dependent on multiple r-proteins and may occur in stages during 30S subunit assembly. The other two r-proteins, S11 and S21, which are in the S15 assembly branch do
not appear to alter the 16S rRNA environment of S15. Thus, the platform proteins S6 and S18, which are directly linked to S15 in the assembly map, play a role in organizing the 16S rRNA environment of S15 (Figure 7). However, these proteins alone are not sufficient for determining the 30S subunit 16S rRNA environment of S15.

Identification of target sites for Fe(II)-S15 within helix 11 of the 5' domain of 16S rRNA are consistent with the positioning of these elements in the 30S subunit. Given the strong footprints of S17 in helix 11 and the many contacts observed between S17 and the 5' and central domains of 16S rRNA in the 30S subunit, it was somewhat surprising that S17 was not responsible for directly positioning helix 11 for cleavage by Fe(II)-S15. Although a direct role could not be identified, S17 may play a role in forming an initial set of interactions that then allows S16 to orient helix 11 such that it is in the vicinity of S15. R-protein S16 extensively footprints both the 5' and central domains of 16S rRNA and contacts both domains in the 30S subunit structure, although these sites are somewhat removed from the Fe(II)-S15 target sites. Additionally, S16 footprints helix 21, which contains the minimal binding site for S8 and crosses the back of the body of the 30S subunit. Since S8 was present in the S16 addition experiments, it is possible that S16 and S8 together play a significant role in orienting helix 11 relative to the central domain and S15 during 30S subunit assembly (Figure 7). A role for S16 in 30S subunit assembly was previously noted; omission of S16 from in vitro 30S subunit reconstitutions greatly affects the rate of 30S subunit formation. In the absence of S16, an inactive assembly intermediate which sediments at 27S is formed and over time this intermediate converts into functional 30S subunits. The role identified here for S16 in developing the 16S rRNA neighborhood of
S15 may involve the relative positioning of major domains of the 30S subunit, which could eventually align even in the absence of S16.

Cleavages in helix 27 in Fe(II)-S15/1°/S16/S5/16S rRNA RNPs occur in the same region as those seen in Fe(II)-S15-30S subunits. This suggests that S5 plays a role in influencing the orientation of the central domain. S5 interacts with 16S rRNA in the 5' and central domains. The S5 contacts in helices 1, 3 and 28 flank those in helix 27 in the secondary structure of 16S rRNA (see Figure 5). Additionally, the 30S subunit structure reveals that S5 is found to bridge the body and the head of the 30S subunit. Thus, by bringing helix 27 proximal to S15, S5 might have a crucial role in orienting all three of these domains. Since binding of S5 depends on S8, this further supports our hypothesis that S8 might influence alignment of these domains relative to one another.

The only cleavages that we have not been able to attribute to r-protein-dependent conformational changes are those in helix 44. These cleavages are the weakest observed in Fe(II)-S15-30S subunits and are the only cleavages that are not within the 45Å range that would be expected from calibration experiments. In RNPs this helix may be mobile, or occupying different positions (see) thus not allowing these weak cleavages to be detected. Also, it is possible that many different factors, likely including S16, are involved in positioning this functionally important element and that it is not positioned until very late in 30S subunit assembly.

Taken together, these results identify the manner in which the 16S rRNA environment of S15 is formed during 30S subunit assembly. Additionally, this approach reveals roles of r-proteins in assembly of specific rRNA elements and this effect can occur at some distance. The farthest distance between the center of mass of an r-protein (S8) and the
16S rRNA elements (helix 26) it orients is found to be 68Å. Therefore, ribosomal proteins can influence 16S rRNA conformations which are quite remotely located. All of our data taken together suggest that individual r-proteins are involved in orchestrating specific 16S rRNA conformational changes. Additionally, these data suggest that r-proteins act at a distance to influence the appropriate folding of 16S rRNA during 30S subunit assembly.

Materials and Methods

Formation of Fe(II)-S15 containing RNPs

Natural 16S rRNA (40pmoles) isolated from natural 30S subunits as described, was pre-incubated at 42°C for 15 minutes in buffer A (20mM K⁺-Hepes (pH 7.6), 20mM MgCl₂). Next, 320pmoles of Fe(II)-S15 (S12C, N36C, K46C, K70C as in) and the required r-proteins for forming either 30S subunits or specific RNPs were added sequentially according to the ordered assembly pathway. For example, 30S subunits were formed by adding 320pmoles of Fe(II)-S15 and 320pmoles of the remaining 1° binding proteins and incubated for 30 minutes at 42°C. The 2° binding proteins (320pmoles) were then added followed by another 30 minutes incubation at 42°C. Subsequently, 320pmoles of the 3° binding proteins were added and the reactions were incubated for 30 minutes at 42°C. RNPs were formed as previously described. After each set of proteins was added and reactions were adjusted to 330mM KCl and a final volume of 100μL.
Purification of Fe(II)-S15/16S rRNA complexes

*In vitro* reconstituted 30S subunits containing Fe(II)-S15 were purified by sucrose gradient sedimentation analysis as previously described. More minimal RNPs containing Fe(II)-S15 were purified from unbound free protein using gel filtration spin columns as previously described.

Transfer RNA binding

tRNA binding assays were performed essentially as described by Culver and Noller using Fe(II)-modified 30S subunits that were purified as described above.

Directed hydroxyl radical probing and primer extension

Fe(II)-derivatized S15 containing 30S subunits and RNPs were probed for 10 minutes and analyzed by primer extension as described by Culver & Noller.

Distance measurements

The program MOLMOL was used to calculate distances between the alpha carbon atom of the natural amino acid at the tethering site within S15 and the phosphate group in 16S rRNA. Also the distances between the center of mass of r-proteins and the cleavage sites that they are responsible for was also calculated using this program.
Acknowledgements

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References


Table 1. Transfer RNA binding of 30S particles with Fe(II)-S15

<table>
<thead>
<tr>
<th>30S subunit</th>
<th>% binding</th>
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<tbody>
<tr>
<td>Wt</td>
<td>100 +/- 14</td>
</tr>
<tr>
<td>C12</td>
<td>91 +/- 19</td>
</tr>
<tr>
<td>C36</td>
<td>81 +/- 10</td>
</tr>
<tr>
<td>C46</td>
<td>90 +/- 11</td>
</tr>
<tr>
<td>C70</td>
<td>92 +/- 25</td>
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Filter binding of tRNA was performed as previously described\textsuperscript{22}
Figure 1: *In vitro* assembly map of the 30S subunit. The primary binding proteins are shown in black; the secondary binding proteins are shown in blue and the tertiary binding proteins are shown in pink. S6 and S18 are shown in a dashed box to indicate they bind as a heterodimer. The assembly map is slightly simplified and modified from the original map\textsuperscript{1;2}.
Figure 2: Components of the 30S subunit. (a) Footprints (black circle) and direct contacts (grey diamond) of S15 shown on the central domain of 16S rRNA. Helix numbers are indicated. (b) Three-dimensional structure of *Thermus thermophilus* (T. thermophilus) 30S subunit. 16S rRNA is shown in light grey; S15 is shown in black with the cysteine substituted sites colored; position 12 is shown in blue, 36 in magenta, 46 in yellow and 70 in green. Other small subunit r-proteins are shown in dark grey. (c) Structure of S15 taken from (b). All figures of 30S subunits were prepared using Ribbons.
Figure 3: Sucrose gradient sedimentation analysis of in vitro reconstituted 30S subunits using Fe(II)-tethered S15 proteins. (a) Natural 16S rRNA and 30S subunits; (b)-(f) 30S subunits reconstituted with natural 16S rRNA and recombinant small subunit r-proteins including (b) wild-type S15 treated with Fe(II)-BABE in a mock reaction, wt-S15 (mock); (c) Fe(II)-C12-S15; (d) Fe(II)-C36-S15; (e) Fe(II)-C46-S15; (f) Fe(II)-C70-S15.
16S/30S

(a) 16S/30S

(b) wt-S15 (mock)

(c) Fe(II)-C12-S15

(d) Fe(II)-C36-S15

(e) Fe(II)-C46-S15

(f) Fe(II)-C70-S15
Figure 4: Directed hydroxyl radical cleavage of 16S rRNA from Fe(II)-S15 in RNPs and 30S subunits detected by primer extension. Complexes all contain natural 16S rRNA and Fe(II)-S15. (a)-(g) Fe(II)-S15-30S; gradient purified 30S subunits containing Fe(II)-S15. (a) Fe(II)-S15/17S16/16S rRNA; 16S rRNA RNP containing the Fe(II)-S15, remaining primary binding proteins and S16. (c) & (d) Fe(II)-S15/S6+S18/16S rRNA; 16S rRNA RNP containing Fe(II)-S15 and S6+S18. (f) Fe(II)-S15/17S16/S5/S12/16S rRNA; 16S rRNA RNP containing Fe(II)-S15, the remaining primary binding proteins, S16, S5 and S12. Fe(II)-S15/17S16/S5/16S rRNA; 16S rRNA RNP containing Fe(II)-S15, the remaining primary binding proteins, S16 and S5. A, G, sequencing lanes; wt, Fe(II)-BABE treated wild-type S15; C12, Fe(II)-C12-S15; C36, Fe(II)-C36-S15; C46, Fe(II)-C46-S15; C70, Fe(II)-C70-S15. Bars at the right indicate regions of 16S rRNA cleavage.
**Figure 5:** Directed hydroxyl radical cleavage sites mapped on the secondary structure of 16S rRNA. (a) Secondary structure of 16S rRNA with regions containing Fe(II)-S15 cleavage sites highlighted. These highlighted domains are expanded in (b)-(e). (b) sites cleaved from Fe(II)-S15 in Fe(II)-S15/16S rRNA. (c)-(e) sites cleaved from Fe(II)-S15 in 30S subunit. The target nucleotides are marked by colored circles corresponding to the similarly colored cysteine-substituted sites on S15 shown in Figure 2. Concentric rings of color represent cleavage of a nucleotide from Fe(II) tethered to more than one position. The 3' end of Helix 24 is targeted by Fe(II) tethered to all the four positions in S15 and is represented by adjacent concentric rings. Some helix numbers are given.
**Figure 6:** Directed hydroxyl radical cleavage sites mapped on to the three-dimensional structure of *T. thermophilus* 30S ribosomal subunit. (a) Three-dimensional structure of 30S subunit indicating all the cleavage sites from Fe(II)-S15 in Fe(II)-S15-30S. The boxed region includes all sites that are cleaved by Fe(II)-S15 and is expanded in (c)-(f) and some helix numbers are given; (b) 180° rotation of (a); (c) Expanded view of sites cleaved from Fe(II)-S15 in Fe(II)-S15-30S; (d) 180° rotation of (c); (e) Sites cleaved from Fe(II)-S15 in Fe(II)-S15/16S rRNA complex; (f) 180° rotation of (e); The position of the phosphate backbone that is cleaved is marked in color corresponding to the Fe(II) attachment site (see Figure 2). Concentric rings of color represent cleavage on a nucleotide from Fe(II) tethered from more than one position.
**Figure 7:** Proteins involved in determining the 16S rRNA environment of S15 within the 30S subunit. (a) Three dimensional structure of *T. thermophilus* 30S subunit $^{16}$ with 16S rRNA in light grey, S15 in black and the proteins responsible for conformational changes in the RNA environment of S15 are colored as follows: S5 in dark green; S6+S18 in light purple since they bind as a heterodimer and cleavages due to individual protein could not be distinguished; S8 in orange; S16 in light blue. Cleavage sites from Fe(II)-S15 in Fe(II)-S15-30S marked in the colors corresponding to the protein responsible for that cleavage. (b) 180° rotation of (a). (c) & (d) Expanded regions of secondary structure of 16S rRNA as highlighted in 5(a) showing cleavage sites from Fe(II)-S15 in Fe(II)-S15-30S marked in the colors corresponding to the protein responsible for that cleavage as shown in Figure 7a and b.
CHAPTER 4: GENERAL SUMMARY AND CONCLUSIONS

"What we have at present are a few snapshots, and ultimately what we would like is a movie of the ribosome in action".

-Harry Noller, 2001

General summary

This study has shed light on the rearrangements that occur in the 16S rRNA during the assembly of the small subunit of *E. coli* ribosome. It also demonstrates the use of directed hydroxyl radical probing to address the conformational changes and cooperative movements brought about by the binding of different components during the formation of the 30S subunit. Using the recombinant reconstitution system, RNPs were constructed that represent different stages of assembly. Monitoring the RNA neighborhood of Fe(II)-S15 in these complexes has given insights into reorientation of the RNA elements as a result of protein binding events. Role of r-proteins that organize the RNA environment of S15 are brought to light.

As a proof of principle experiment to validate the new approach, hydroxyl radical cleavage patterns were generated in two simple RNPs: Fe(II)-S15/16S rRNA and Fe(II)-S15/S8/16S rRNA. The 16S rRNA environment of S15 in these complexes was found to be distinct indicative of conformational change in the presence of S8. Although S8 is found near the platform, assembly map suggests its role in determining the binding of an entirely different set of proteins that interact with the body of the subunit. This study has brought to light the role of S8 in folding of the platform. By virtue of its location in the body according
to the crystal structure, S8 might play a role in orienting platform with the body of the subunit.

Directed hydroxyl radical probing of Fe(II)-S15-30S has revealed that apart from the helices in the central domain, penultimate stem and helix 13 of the body also form part of the RNA environment of S15 in 30S subunit. Rearrangement of these helices results in the proper orientation of platform with respect to other domains in the subunit. Investigating into the roles of other r-proteins in organizing the RNA environment of S15 has reflected on the synergy between different domains during assembly. Apart from the platform proteins S6 and S18 that bring about conformational change in the upper part of helix 22, other r-proteins including S5 and S16 that bind to the 5' domain of the RNA play a role in arranging the RNA elements surrounding S15. Role of S6 and S18 in this process is not surprising given that these proteins interact with the platform and are directly dependent on S15 to bind to 16S rRNA. However role of r-proteins that interact with the body in orienting the platform indeed is unexpected. These results indicate that formation of 30S subunit follows a much more complex pathway than what is described by the in vitro assembly map and domain organization studies. Proteins binding to one domain have influence on the folding and orientation of another domain with rest of the macromolecular complex. Most of the r-proteins effect long range rearrangements. While S6 and S18 orient helical elements close to their binding site, S16 and S8 rearrange RNA regions at a distance. This likely occurs by propagation of the conformational change in the RNA near the binding site to the elements that are far away.

Apart from bringing to light the rearrangements that occur in the RNA elements during 30S subunit assembly this study also demonstrates the use of directed hydroxyl
radical probing \(^4\) to address conformational changes in any macromolecular complex made up of nucleic acids and proteins. Reorganization of the nucleic acid elements can be followed during assembly or ligand binding events by employing one of the constituents as the probe.

**Future direction**

**Employing other r-proteins or ligands as probes**

The current study has employed r-protein S15 as the probe to understand reorientation of 16S rRNA elements in and around the platform. Other primary r-proteins such as S7 and S4 can be converted into probes to address conformational changes in the head and body respectively. The ability to attach Fe(II)-loaded linker to tRNA can be exploited to use the RNA ligand as probe and get insights into the rearrangements that occur during subunit association and translation.

**Temperature dependence of reconstitution and r-protein binding mechanism**

*In vitro* reconstitution of 30S subunit occurs at non-physiological temperature, i.e. 42°C \(^5\); \(^6\). This temperature dependence has been employed in the past to understand the steps involved in binding of one of the primary binding proteins, S4. These studies can be extended to dissect the mechanism of S15 binding. Construction of S15/16S rRNA complexes at different temperatures and analyzing protein-RNA interaction in these RNPs with base-specific probes \(^7\) and hydroxyl radicals \(^8\) might shed light on S15 binding to its two distinct binding sites on 16S rRNA. In addition, these studies can also be used to address co-transcriptional assembly by dissecting the steps in binding of r-proteins like S8 \(^9\); \(^10\) that have interaction sites far away from one another.
Conformational change in S15 upon RNA binding

In this study, conformational changes that occur in the 16S rRNA upon protein binding have been thoroughly investigated using S15 as the probe. However it is yet to be seen if the protein undergoes a conformational rearrangement concomitant with RNA binding suggesting induced fit. As discussed in chapter 1, NMR \(^{11}\) and X-ray structures \(^{12}\) of S15 show some differences that have been hypothesized to play a role in RNA binding. Currently, a wide range of approaches including FRET and crosslinking have been employed to address conformational change in S15 upon interacting with its RNA ligands. In FRET studies, tryptophan has been introduced in the protein and this endogenous amino acid acts as the donor. A fluorescing dye that acts as the acceptor is attached to unique positions via cysteines. The transfer between the donor and acceptor in the presence and absence of RNA are being monitored. A change in the energy transfer is indicative of conformational change in the protein.

Crosslinking the N-terminal helix with an amino acid in the body of the protein and investigating into reconstituting ability of the crosslinked protein might shed light on if the conformational flexibility of this helix is important for RNA binding and thus, 30S subunit assembly. This work is currently ongoing.

References


APPENDIX. ELUCIDATION OF THE STEPS IN BINDING OF S15 TO THE CENTRAL DOMAIN OF 16S rRNA

Summary

S15 is one of the six primary binding proteins that bind to 16S rRNA independently. S15 interacts with the central domain of the RNA and its binding has been characterized extensively by footprinting and structural studies. According to footprinting analysis, S15 interacts with the backbone of the RNA at the three way helical junction between helices 20-22 and near the purine rich internal loop in helix 22. Base-specific probing reveals footprints of S15 in helices 22 and 23b. Temperature dependence of in vitro reconstitution and interaction of S15 with the RNA has been exploited to dissect the steps involved in the binding of the r-protein to 16S rRNA. Interaction of S15 with the RNA backbone at the three-way helical junction is observed when the RNA-protein complex is formed at 0°C. Base-specific footprints of S15 near the internal loop in helix 22 are also seen at 0°C. However, base-specific footprints in helix 23b are seen only at elevated temperature i.e. 42°C suggesting that this might be a secondary effect that occurs after the interaction of S15 with its principle binding sites. The secondary effect leading to a possible conformational change might facilitate the binding of S6 and S18 that interact with this region of the RNA.

Introduction

The primary binding ribosomal protein S15 initiates the folding of central domain of 16S rRNA to form the platform and governs the association of four other proteins in this
process. The interaction of S15 to 16S rRNA has been characterized extensively by structural and biochemical studies. The minimal binding site of S15 in the rRNA has been confined to the three-way helical junction of helices 20, 21 and 22 and the purine-rich internal loop in the upper part of helix 22. Crystal structure of S15 in complex with its minimal binding site in 16S rRNA supports the earlier observation of two closely spaced and yet distinct binding sites for S15 on the rRNA. Footprinting analysis employing base-specific probes and solution hydroxyl radicals has also shed light on the nucleotides of 16S rRNA that are involved in the interaction with the r-protein. S15 is one of the r-proteins with limited and localized footprints. Base-specific footprints are confined to helix 23 and the purine-rich internal loop in helix 22. Majority of these footprints are the result of protections of guanosines. Only one adenosine is protected in the presence of S15. Solution hydroxyl radical footprints are located predominantly in the three-way helical region and are 3' staggered indicative of minor groove interaction of the protein with the rRNA. Binding of S15 to the RNA despite removal of helix 23 suggests that the footprints observed in helix 23 are likely to be the result of secondary effect. In addition the distance constraints mainly due to its position in the neutron map point out that it is impossible for the r-protein to interact with both the 740 and 730 elbow regions simultaneously. Hence, the footprints in helix 23 are likely due to conformational change that results from the binding of S15 and this rearrangement might aid in the interaction of other central domain binding proteins such as S6 and S18.

In this study, interaction of S15 with 16S rRNA has been revisited in order to dissect the steps in binding of this protein to the RNA. Earlier, temperature dependence of in vitro reconstitution was exploited to understand the mechanism of interaction of the ribosomal
protein S4 to its different binding sites in 16S rRNA. S4/16S rRNA complex was reconstituted in ice and 42°C. Analyzing the difference in the footprints of S4 at these temperatures indicates that this protein recruits additional sites of interaction in the 16S rRNA following its initial binding. On the same lines, in order to dissect into the mechanism of S15 binding, S15/16S rRNA complexes are formed on ice and 42°C and the footprints from base-specific probes and hydroxyl radicals monitored at the two conditions. Appearance of the footprints at the three-way helical junction and purine-rich internal loop at 0°C indicates that S15 interacts with both its binding sites as a one-step process. Temperature dependence of the helix 23 footprints shows that the nucleotides in this region undergo a change in reactivity as the second step following S15 binding.

Results

Effect of temperature on the interaction of S15 with 16S rRNA probed using kethoxal

The reactivity of 16S rRNA in its naked form and in complexes with S15 has been examined towards base-specific probe, kethoxal. S15/16S rRNA complexes were formed at 0°C and 42°C. In order to understand the effect of change in temperature on the binding of S15, the RNA-protein complexes were also formed at 0°C and transitioned to 42°C. Analysis of base-specific footprints reveals that protections in the purine-rich internal loop of helix 22 are temperature independent. The guanosine at 745 gets protected upon binding of S15 even when the complex is formed on ice (Figures 1(a) & 2). However, the footprints at the elbow region of helix 23 show temperature dependence. The guanosines at the 730 region do not show protection at 0°C but are strongly protected at elevated temperature (Figures 1(a) & 2). Heat activating the rRNA and pre-incubation of S15 did not substitute for the temperature
dependent effects observed in the footprints of S15/16S rRNA complex. This confirms that the additional footprints are not the result of renaturing of either S15 or 16S rRNA.

Dimethyl sulfate hasn’t been employed in this study to address the change in reactivity of adenosines since S15 footprints only one adenosine which is in the elbow of helix 23.

**Probing S15/16S rRNA complexes using hydroxyl radicals**

Susceptibility of the sugar-phosphate backbone is monitored in the naked rRNA and S15/16S rRNA complexes formed at different temperatures. Protection of the backbone from hydroxyl radical attack due to the binding of S15 on ice and at 42°C is analyzed. All the nucleotides that are protected by S15 from hydroxyl radicals are located within the minimal binding site of the protein. Treatment of naked 16S rRNA with hydroxyl radicals results in a nearly uniform cleavage pattern (Figures 1(b) & (c), lanes 4 & 5; Figure 2). Upon binding of S15, elements in the three-way helical junction at nucleotides 640 and 750 are protected at both 0°C and 42°C (Figures 1(b), 1(c) & 2). Strong protection is observed in this region and identical results are observed whether the complexes are formed under low and high temperature conditions. Therefore, accessibility of riboses does not change as part of the heat-dependent conformational change in the S15/16S rRNA complex. Thus, interaction of S15 with 16S rRNA with the sugar-phosphate backbone occurs in a temperature independent manner similar to what is observed for r-protein S4. Temperature-dependent changes in the S15 ribonucleoprotein particle are due likely to base-base or base-protein interactions as suggested by the temperature dependence of base-specific footprints at helix 23.
Discussion

The results from the above temperature dependent footprinting studies shed light on the mechanism of binding of r-protein S15 to 16S rRNA. The principle interactions of S15 at its two binding sites \(^8\) occur in a single step as indicated by the temperature independent nature of the footprints in this region. The base-specific footprints at 730 region are seen only at elevated temperature indicating that these S15-dependent changes occur as the next step after the binding of S15 to its primary sites. Since hydroxyl radicals attack the ribose moiety independent of the secondary structure and double stranded region in the RNA, protection of the backbone from the radicals is likely due to the direct interaction of the protein with that RNA element. However, footprints from base-specific probing \(^10\) bring to light both direct protein-RNA interaction and indirect effects such as conformational change or protein-dependent base pairing. Therefore, absence of hydroxyl radical footprints in helix 23 by S15 \(^11\) and this helix not being part of S15 minimal binding site indicate that this region is not likely a direct interaction site for S15 \(^10; 11\). Base-specific as well as backbone footprints are observed at 0°C in the primary binding sites of this r-protein indicating that S15 contacts with both RNA bases and backbone occur in the first step. Localized interactions and closely spaced binding sites of S15 \(^8\) further support this mechanism. Upon binding to these sites, the r-protein likely brings about conformational changes in the RNA elements of helix 23 as indicated by the base-specific footprints observed in these elements. These rearrangements might result in the formation of binding sites for platform protein such as S6, S18 that directly depend on S15 to interact with the rRNA.

As demonstrated in the above study with S15 and previous work with r-protein S4 \(^11\), this temperature sensitive approach can be extended to understand sequential addition of
proteins during ribosome assembly. Footprints of r-protein S8 are found on two distantly spaced helices, 21 and 25 in the central domain, where helix 21 is alone forms the minimal binding site. Secondary binding protein S16 has its footprints distributed across different domains including the 5', central and 3' minor domains. Given the small size of these proteins it is highly unlikely that interaction at RNA elements so far away from each other occurs as a single step process. Temperature dependence of reconstitution can be employed in these cases to investigate into the footprints that are the result of first step interaction with the protein and which ones occur following the primary binding step. Earlier work has shed light on the possible coupling of transcription of 16S rRNA and assembly of the 30S subunit. Investigating the mechanism and sequential nature of r-protein binding to 16S rRNA can give further insights into this process. Among the widely distributed footprints, the likely temperature independent nature of those located towards the 5' end and heat activated reactivity change of nucleotides in the 3' region would support the co-transcriptional model.

Materials and methods

Construction of S15/16S rRNA complexes

Natural 16S rRNA (40pmoles) isolated from natural 30S subunits as described was pre-incubated at 42°C for 20 minutes in buffer A (20mM K⁺-Hepes (pH 7.6), 20mM MgCl₂) and then cooled on ice for 15 minutes prior to the addition of S15. Complexes between 16S rRNA and S15 were formed by incubating 160pmoles of the protein with the RNA at 0°C and 42°C for one hour. S15-16S rRNA complexes were also formed at 0°C for 30 minutes and shifted to 42°C for another 30-minute incubation. Control reactions with 16S rRNA
alone incubated at 0°C and 42°C were also setup. These complexes were stabilized on ice for 15 minutes before the addition of chemical modification reagents.

**Base-specific modifications and hydroxyl radical footprinting**

Chemical modification reagents were added to naked 16S rRNA and S15/16S rRNA complexes as described\(^\text{19,20}\). Footprinting using kethoxal, which modifies the N-1 of G, was performed on ice for approximately 60 minutes. Sugar-phosphate backbone was monitored by hydroxyl radical probing for approximately 10 minutes.

**References**


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Figure 1: Primer extension analysis of naked 16S rRNA and S15/16S rRNA complexes formed at different temperatures using (a) kethoxal and (b) & (c) hydroxyl radical. A, G, sequencing lanes; 16S unmod., unmodified 16S rRNA, 16S mod. 0°C, 16S rRNA without protein incubated at 0°C; 16S mod. 42°C, 16S rRNA without protein incubated at 42°C; 16S rRNA + S15 0°C, S15/16S rRNA complex formed at 0°C; 16S rRNA + S15 0°C -> 42°C, S15/16S rRNA complex formed first at 0°C and transferred to 42°C for another 30-minute incubation; 16S rRNA + S15 42°C, S15/16S rRNA formed at 42°C. Arrows in (a) indicate sites of protection from kethoxal. Bars at the right in (b) & (c) indicate regions of 16S rRNA cleavage.
Figure 2: Central domain of 16S rRNA with base-specific (black dots) and hydroxyl radical (black diamonds) footprints of S15 mapped. (a) depicts footprints of S15 on 16S rRNA at 0°C. (b) depicts footprints of S15 on 16S rRNA at 42°C.
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