Published in final edited form as:

Curr Opin Chem Biol. 2008 December ; 12(6): 667–673. doi:10.1016/j.cbpa.2008.09.024.

RNA folding and ribosome assembly

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Summary

Ribosome synthesis is a tightly regulated process that is crucial for cell survival. Chemical footprinting, mass spectrometry and cryo-electron microscopy are revealing how these complex cellular machines are assembled. Rapid folding of the rRNA provides a platform for protein-induced assembly of the bacterial 30S ribosome. Multiple assembly pathways increase the flexibility of the assembly process, while accessory factors and modification enzymes chaperone the late stages of assembly and control the quality of the mature subunits.

Introduction

Cells must produce new ribosomes in order to grow, yet ribosome biogenesis places heavy demands on the cell's metabolic resources [1]. Moreover, the finished ribosomal subunits must function perfectly to avoid poisoning protein synthesis. Given these stiff requirements for efficiency and quality, it is not surprising that ribosome biogenesis is tightly controlled.

The simplest ribosomal complex, the bacterial 30S subunit, contains a ~1530 nt 16S rRNA and at least 20 proteins, with a combined mass approaching 1 MDa [2]. How the components of these large complexes organize themselves remains a daunting problem, with implications for bacterial pathogenesis, aging, and a host of other diseases [3].

Early insights into the mechanism of 30S ribosome assembly came from reconstitution of active subunits from the rRNA and protein components by Nomura and co-workers [4]. They showed that the six primary assembly proteins bind the naked 16S rRNA, while secondary assembly proteins require one or more primary assembly proteins, and the tertiary assembly proteins bind after a temperature-dependent conformational step [5,6]. The hierarchy of protein binding leads to cooperative assembly, ensuring each complex forms completely. This cooperativity mostly arises from structural changes in the 16S rRNA induced by the progressive addition of proteins [7,8].

Although in vitro reconstitution of 30S subunits established the principle of self-assembly, many questions remain, such as how protein binding is coupled to rRNA folding, whether some intermediates are obligatory, and how fidelity is maintained given the structural complexity of the ribosome. Moreover, reconstitution of the mature rRNA cannot recapitulate the links between assembly, transcription and processing of the pre-rRNA which exist in the cell and which make assembly more cooperative [9].

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This review will discuss recent insights into the folding, assembly and maturation of bacterial 30S ribosomes, derived from sophisticated application of molecular genetics, biochemistry and structural biology. Readers are encouraged to consult other reviews for a description of early work [7], biophysical methods [10], and ribosome biogenesis in yeast [11,12].

Folding of the 16S 5' domain

Crystal structures of the large and small ribosomal subunits revealed that the rRNA forms the core of the ribosome [2], as predicted from the deep conservation of the rRNA among all known organisms and its direct interactions with substrates [13]. Small angle neutron scattering studies showed that the deproteinized 16S rRNA is as compact as it is in the 30S ribosome, evidence that the naked rRNA retains some self-organization [14]. If the ribosome's architecture is defined by interactions within the rRNA (rather than between the rRNA and the proteins), to what extent is hierarchy of protein binding encoded by the rRNA?

This question was addressed by probing the folding pathway of the 5' domain of the *E. coli* 16S rRNA with hydroxyl radical footprinting, which measures the solvent accessibility of the RNA backbone [15]. The 5' domain of the 16S rRNA, which forms the body of the 30S subunit, is particularly rich in RNA interactions, and was observed to retain a kernel of structure after the rRNA was stripped of proteins [16]. Because this domain is the first to be transcribed, stable RNA interactions in this region might plausibly nucleate assembly.

Remarkably, all of the expected tertiary interactions in the 5' domain formed in the absence of proteins [15]. However, interactions between helix 15 and 17, which create the binding site for the secondary assembly protein S16, required 4 mM MgCl₂ to form. Thus, the 5' domain RNA can fold independently, but the ribosomal proteins are needed to stabilize the RNA tertiary structure in physiological Mg²⁺ concentrations (< 4 mM).

Time-resolved hydroxyl radical footprinting using a synchrotron X-ray beam showed that nearly all the tertiary interactions formed within 20 ms in 20 mM MgCl₂ [15]. Half the RNA population, however, was kinetically trapped in intermediates lacking the proper contacts between helices 15 and 17, due to mispairing of nucleotides joining helices 6, 7 and 12. The proportion of slow folding RNA (~1 min) was not reduced when the RNA was allowed to fold during transcription, suggesting that misfolding is an intrinsic feature of the RNA (T. Adilakshmi and S.W., unpublished). Thus, another potential role of the ribosomal proteins is to suppress misfolding of the rRNA or to facilitate reorganization of folding intermediates.

The 16S 5' domain exhibits common features of RNA folding reactions [17,18]. Once the secondary structure is established, many structured RNAs first collapse into compact intermediates (1-10 ms), in which the helices align and begin to form tertiary interactions, then rearrange more slowly to the native conformation (10 ms – 100 min). While some molecules fold directly to the native state, others pass through kinetically trapped, misfolded intermediates [19]. As discussed below, the need to refold the rRNA results in temperature-dependent steps of assembly and the cold sensitive phenotypes of assembly deficient strains. The co-existence of folding pathways, however, may explain the partial redundancy of certain assembly factors.

In general, long RNAs have many chances to mispair. Thus, it is not surprising that the 560 nt 5' domain misfolds, but rather, that it folds so well. It is interesting to consider how eons of natural selection may have sculpted the rRNA to be easily folded. Except for loop-loop and loop-helix interactions which reinforce the bundle of helices surrounding the spur (helix 6), the 5' domain is only one helix thick [20]. The core of the 5' domain also lacks pseudoknots, which are found in many ribozymes but commonly misfold.

Cooperative binding of proteins and RNA

Although the rRNA defines the architecture of the ribosome, protein-rRNA interactions in the central domain illustrate how successive proteins stabilize the 3D interactions that encoded by the rRNA [8]. The thermodynamic cooperativity between protein binding reactions in the central domain is almost entirely explained by a reduction in binding entropy (ΔS), consistent with greater organization of the RNA [21]. This hierarchy is not absolute, however, as the degree of cooperativity can vary among species [21]. This plasticity may reflect the fact that the rRNA is predisposed toward the correct structure [8].

The 30S proteins make relatively few base-specific contacts with the rRNA, but recognize the shape of the folded RNA [22]. Co-folding of the RNA and the protein, in which both partners change their structure in the complex, can increase the specificity of assembly, as a large number of favorable intermolecular contacts are needed to offset the energetic cost of folding regions that are disordered in the free protein or RNA [23].

Evidence for co-folding first came from temperature-dependent conformational changes in the S4-16S rRNA complex that were only observed when the protein and RNA were incubated together at 42 °C [24,25]. More recently, chemical footprinting revealed temperature-dependent remodeling of S7 and S8 complexes [26]. Protein S7 induces extensive conformational changes in the 16S 3' domain [27], and along with S4, nucleates 30S assembly [28]. Protein S8 is important for assembly of the central domain [29]. It remains to be proven that remodeling of ribosomal complexes involves restructuring of the polypeptide, although S4 and S7 are partially disordered in solution [30-32].

Kinetic pathway of 30S assembly

To understand how ribosomes self-assemble, one must know how the components associate with each other in real time. Talkington et al. [33] devised a clever mass spectrometry method for measuring the rates at which individual proteins join the 30S complex. After the assembly is begun by adding 15N-labeled proteins to the 16S rRNA, labelled complexes are chased with an excess of 14N-labeled proteins. The amount of each 15N-labeled protein bound during the labeling pulse is measured by mass spectrometry, from which the rate constants for protein binding are calculated. Only productively bound 15N-labeled proteins are counted, as weakly bound proteins are washed out by the chase.

The protein binding kinetics roughly correlate with the equilibrium assembly map, and with the 5' to 3' order of 16S folding observed previously [34]. At temperatures below 15 °C, reconstitution stalls at the RI intermediate (21S), which lacks the tertiary assembly proteins [5,6]. Activation of this complex at 42 °C results in a remodeled complex RI* (26S), which can add the remaining proteins to form the 30S subunit. If addition of the tertiary proteins is limited by the same molecular step, they should bind with the same kinetics. Surprisingly, neither the binding rates at 15 °C nor the activation enthalpies ($\Delta H^\ddagger$) were the same [33], challenging the notion that RI represents a single bottleneck for assembly. Parallel assembly pathways allow ribosome biogenesis to bypass steps blocked by a mutation or protein deficiency, as demonstrated by the ability of *E. coli* to survive without protein S15, albeit with long doubling times and abnormal levels of immature 30S complexes [35].

Assembly factors monitor late steps in 30S biogenesis

Given the myriad ways in which 30S assembly can go awry, it is remarkably successful. In the cell, assembly is coupled to transcription, allowing each domain to fold before the next domain is transcribed. The fidelity of assembly is also increased by accessory factors, which bind to

immature complexes but not active subunits [9]. Recent cryo-electron microscopy structures show how assembly factors bind the 30S subunit.

Many assembly factors associate with regions of the 16S that change conformation in the late stages of assembly, where the head, body and platform come together. Holmes and Culver [36] used chemical footprinting to detect conformational changes in the 16S rRNA between the transition from RI to RI* and to the mature 30S complex. The temperature-dependent change from RI to RI* involved formation of the central pseudoknot (helix 2) that joins the 5', central and 3' domains, as well as many interactions in the 3' domain (head and neck) [36]. These vulnerable regions of the complex may especially rely on accessory factors for their assembly.

At least one assembly protein, RimM, plays a direct role in maturation of the 30S head domain [37]. RimM binds protein S19, and *rimM* strains are partially suppressed by mutations in S13 and S19 [38]. Suppressor mutations were also found in the 16S rRNA adjacent to the S19 binding site, suggesting that RimM senses the conformation of the rRNA [38]. Three suppressor mutations map to helix 31, which organizes the center of the 3' domain. Two bases in helix 31, C972 and G973, change conformation between RI to RI* and again from RI* to 30S [36]. The other suppressor mutation lies at the tip of helix 33b where it contacts helix 32. Helices 33-33b form the "beak" of the 30S ribosome, another region of the 3' domain that folds late in assembly [36].

Another assembly factor, RbfA, is a small KH-domain cold-shock protein that was first isolated as a multi-copy suppressor of the cold sensitive mutation C23U in helix 1 of the 16S rRNA [39]. As for *rimM*, deletion of *rbfA* results in accumulation of 17S rRNA and immature 30S subunits [37]. A recent cryo-EM model showed that RbfA wedges between helix 44 in the 3' minor domain and helix 28 in the neck of the *Thermus thermophilus* 30S ribosome, putting three of its surfaces in contact with the rRNA [40]. Helices 44 and 28 are adjacent to helix 1 and the central pseudoknot (helix 2), and form critical interactions with mRNA and tRNA substrates. Remarkably, RbfA forces helix 44 and helix 45 out of their normal position in the 30S subunit, closing the decoding site and removing the anti-Shine-Dalgarno sequence from the mRNA binding channel [40]. Thus, RbfA could facilitate refolding or allow more time for assembly by maintaining the pre-30S complex in an inactive state.

A previous cryo-EM reconstruction revealed that Era GTPase binds the opposite face of helix 28 from RbfA, between the 30S head and the platform [41]. Depletion of Era also results in unprocessed 17S pre-rRNA, while overexpression suppresses the cold-sensitive effects of $\Delta rbfA$ [42], suggesting these factors act in the same pathway. It is now clear that GTPases play important roles in ribosome biogenesis, in both bacteria and eukaryotes [43]. In yeast, the Bms1 GTPase recruits a putative processing enzyme, Rcl1p, to immature 40S complexes in conjunction with U3 snoRNP [44].

Modification enzymes: keepers of the gate?

Recent work suggests that dimethylation of A1518 and A1519 in helix 45 by KsgA methylase provides a final check on the quality of 30S assembly [45]. KsgA methylates translationally inactive, but not active, 30S subunits [46]. A combination of tethered hydroxyl radical footprinting and solution probing was used to dock KsgA on helices 44, 45, and 28, which are all near the decoding site [45]. KsgA must swing helix 45 out of position in order to access A1518 and A1519, as these bases interact with the minor groove of helix 44 in mature subunits. In doing so, KsgA would prevent immature subunits from entering the translation cycle, where they would compromise translational fidelity [45]. The notion that modification enzymes are in fact assembly factors is supported by experiments in yeast, showing that the

methyltransferase activity of Dim1 (KsgA) and Bud23 18S methylases is dispensable although the proteins themselves are not [47,48].

Protein modifying enzymes also serve as “gatekeepers” to the polysome pool. The RimJ acetylase suppresses defects in 30S assembly and translational accuracy arising from an amino acid substitution in protein S5 (G28D) [49]. In yeast, phosphorylation of protein S3 regulates 40S assembly [50]. Protein S3 is a tertiary assembly protein which binds helix 33 in the 3' domain, and pre-40S complexes lacking Rps3p also lack the protruding beak normally visible in electron micrographs [50]. Phosphorylation of Rps3p by Hrr25 kinase induces dissociation of Rps3p from pre-40S ribosomes, while subsequent dephosphorylation of Rps3p leads to its stable incorporation into the 40S ribosome [50]. Protein phosphorylation could conceivably couple conformational changes in the head domain to nuclear export or further processing reactions.

Conclusion

Biophysical studies are producing an increasingly clear picture of how interactions between the ribosomal proteins and the rRNA drive self-assembly of the subunits. Nonetheless, the sheer complexity of the ribosome and the enormous demands on its function push the principle of self-assembly to its limit. Thus, the present challenge is to not only understand the physical process of rRNA folding and protein recognition, but how this process is regulated and inspected by accessory factors. A key issue is whether the assembly intermediates visible in suboptimal conditions mature into function 30S subunits, or whether these are dead-end complexes. The vastly more complex array of processing steps and assembly factors in eukaryotes may serve to control both the temporal and spatial distribution of ribosomal complexes [51,52], linking ribosome biogenesis even more firmly to the capacity for protein synthesis, the quality of translation, and the rate of cell growth.

Acknowledgements

The author thanks the NIGMS for financial support (GM60819).

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Figure 1. Folding of the 16S rRNA 5' domain without proteins

The tertiary structure of the rRNA (nt 21-562) was probed by hydroxyl radical footprinting [15]. a. At equilibrium, the interactions were stable in 120 mM NH₄Cl (blue), low MgCl₂ ([Mg²⁺]_{1/2} = 0-2.5 mM; purple), or high MgCl₂ ([Mg²⁺]_{1/2} = 4-8 mM; green). b. In 20 mM MgCl₂ at 37 °C, many interactions form 60-90% in 20 ms (blue); the lower subdomain folds 40-50% in 20 ms and 30-60% in 30-60 s (orange).

Figure 2. Kinetics of 30S assembly by pulse-chase mass spectrometry

a. Nomura assembly map is colored by the protein binding rates at 37 °C: red, • 20 min⁻¹; orange, 8.1-15 min⁻¹; green, 1.2-2.2 min⁻¹; blue, 0.38-0.73 min⁻¹; purple, 0.18-0.26 min⁻¹. b. 30S subunit from *T. thermophilus* [20], colored as in a. Reprinted from [33] with permission.

Figure 3. Structural changes in the 16S rRNA during late steps of assembly

For structure mapping, reconstitution intermediate (RI) was formed from 16S rRNA plus recombinant S4-S9, S11-S13, S15-S20 at 4 °C, then shifted to 42 °C (RI*), and chased into 30S complexes with the addition of tertiary assembly proteins (S3, S3, S10, S14, S21) [36]. b. Structural differences between RI and RI* determined by dimethylsulfate base modification (red) and hydroxyl radical cleavage (light blue) [36]. N6,N6-dimethyl A1518 and A1519 products of KsgA are shown in green; blue triangles indicate $\Delta rimM$ suppressors [38].

Figure 4. Binding sites of RbfA and Era on the 30S ribosome

a. Superposition of cryo-EM reconstructions showing the locations of RbfA (red) [40] and Era (magenta) [41]. b. Both assembly factors interact with helix 28 (green) of the 16S rRNA. Models were obtained by docking the crystal structure of the 30S subunit [20] into the cryo-EM density map. Reprinted from [40] with permission.