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An assembly landscape for the 30S ribosomal subunit

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Abstract

Self-assembling macromolecular machines drive fundamental cellular processes, including transcription, mRNA processing, translation, DNA replication, and cellular transport. The ribosome, which carries out protein synthesis, is one such machine, and the 30S subunit of the bacterial ribosome is the preeminent model system for biophysical analysis of large RNA-protein complexes. Our understanding of 30S assembly is incomplete, due to the challenges of monitoring the association of many components simultaneously. We have developed a new method involving pulse-chase monitored by quantitative mass spectrometry (PC/QMS) to follow the assembly of the 20 ribosomal proteins with 16S rRNA during formation of the functional particle. These data represent the first detailed and quantitative kinetic characterization of the assembly of a large multicomponent macromolecular complex. By measuring the protein binding rates at a range of temperatures, we have found that local transformations throughout the assembling subunit have similar but distinct activation energies. This observation shows that the prevailing view of 30S assembly as a pathway proceeding through a global rate-limiting conformational change must give way to a view in which the assembly of the complex traverses a landscape dotted with a variety of local conformational transitions.

The assembly of the 30S ribosomal subunit is a complex dance of macromolecular folding and binding in which 20 proteins bind to rRNA as it folds, creating a complete particle^{1–3} that is competent to participate in translation of mRNA. Assembly in vitro has shown that secondary structure in the 16S rRNA (local helices) is stabilized by Mg²⁺-containing buffer alone, but tertiary (long-range) folding depends on the proteins⁴. Because protein binding sites are created as the rRNA folds, ribosomal protein binding reports on local rRNA tertiary conformation throughout assembly^{5–9}. A large body of knowledge on the order and mechanism of 30S assembly has thus been amassed by identifying the proteins bound at equilibrium in incomplete assembly reactions^{10,11}.

A slow rate-limiting folding transition has long been inferred from the observation that incomplete particles with an altered sedimentation coefficient (21 S vs. 30 S) form at low temperatures (0–15 °C)^{12,14}. Heating these intermediate particles (reconstitution intermediate, RI) shifts their sedimentation coefficient to 26 S (RI*) and enables them to complete assembly at low temperatures. The RI→RI* transition, thought to be a conformational change in the rRNA, was proposed to be the rate-limiting step of assembly even at higher temperatures, since the apparent concentration independence of the overall assembly rate suggested a unimolecular

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rate-limiting step¹². RI→RI* characterizes the canonical scheme of 30S assembly, which has remained essentially unchanged for 35 years:

The next step in characterizing the mechanism of 30S assembly is to determine the kinetics by which the various proteins bind to the assembling subunit. However, standard methods are not capable of directly monitoring the binding of many proteins simultaneously. We have developed a new method, PC/QMS (pulse-chase monitored by quantitative mass spectrometry), which measures the kinetics of binding the individual proteins during assembly of the whole complex.

A method for studying whole-30S subunit assembly

PC/QMS takes advantage of the ability of mass spectrometry to quantify large numbers of proteins relative to stable isotope-labeled species, an approach widely employed in proteomics^{15,18}. Assembly of 30S subunits is initiated by incubating the 1542-nucleotide *E. coli* 16S rRNA with a mixture of uniformly ¹⁵N-labeled 30S proteins (S2-S21)¹⁹. At various timepoints, the binding of the ¹⁵N-proteins is chased with an excess of unlabeled (¹⁴N) proteins. Completely formed 30S subunits are purified, and the ¹⁵N-:¹⁴N ratio for each protein is determined by MALDI-TOF (matrix-assisted laser desorption/ionization—time-of-flight mass spectrometry)^{20,22}. (Fig. 1a) The ¹⁵N-:¹⁴N ratios can be quantified accurately, as judged by standard curves collected on known mixtures of labelled and unlabeled proteins (Fig. 1b), and the majority of the 30S proteins are observed in a single scan (Fig. 1c). The assay has been validated by measuring the binding rate of the *Aquifex aeolicus* S15 protein to a 16S rRNA fragment using PC/QMS compared with a gel mobility shift assay, as described in the Supplementary Information (Supplementary Fig. S1). Plotting the fractional isotope ratios for a given protein as a function of time produces a progress curve for the binding of that protein during assembly of the whole subunit. In this way, the binding kinetics of all of the ribosomal proteins can be determined in a single experiment.

Protein binding rates match the existing 30S assembly map

Under standard conditions (see Methods), similar to those identified as optimal for in vitro assembly¹², the proteins bind with rates distributed throughout two orders of magnitude (Fig. 2a,b,c). The trends in these data correspond well to protein binding rates inferred from the reactivity of 16S rRNA nucleotides to chemical probes over time⁶ and to the binding order revealed by the classical equilibrium experiments of Nomura and colleagues¹⁰ (Fig. 2b). Assembly in vitro maintains the 5'-to-3' directionality and overall protein binding order, including late assembly of the interdomain junction that forms the site of mRNA decoding (Fig. 2c), that is observed in vivo^{6,23}, despite taking place on a mature 16S rRNA rather than on a nascent pre-rRNA transcript^{24,25}.

Folding and binding occur at similar rates

Characterizing the mechanism of 30S assembly requires that the protein binding kinetics be measured under a variety of conditions, and PC/QMS is sufficiently rapid to permit collection of datasets under multiple conditions. To begin probing the nature of the rate-limiting steps of assembly, we varied the concentration of rRNA and proteins in the assembly reaction. At one extreme, if binding is the rate-limiting step for a particular protein, the binding rate should be directly proportional to the concentration. If, on the other hand, a unimolecular folding event is rate-limiting, the rate should be insensitive to concentration. Instead, the intermediate case is observed for many proteins, where the observed protein binding rates are weakly affected by concentration (Fig. 3), which indicates that RNA folding and protein binding occur at similar rates. All of the proteins observed here exhibit some degree of concentration dependence in their binding rates, so folding does not appear to be rate-limiting for any of them.

The temperature dependence of the binding rates reveals many rate-limiting transitions

In order to characterize assembly intermediates, we measured protein binding rates at low temperature, where RI has been found to accumulate. The ^{15}N -protein pulse was performed at low temperature and then the temperature was restored to the optimum (40°C) upon adding the ^{14}N -chase. Consistent with previous measurements of overall assembly rates¹², protein binding is slow at 15°C (Fig. 4a), requiring more than two days to proceed to completion. Unexpectedly, none of the proteins are disproportionately slowed compared to the others, and none plateau at a low extent of binding, which initially seemed to be inconsistent with stalling of assembly at a 21S intermediate (RI).

The standard $\text{RI} \rightarrow \text{RI}^*$ mechanism, whereby assembly stalls at the 21S intermediate at low temperatures, implies that the late proteins have much lower rates of binding than the early proteins at the low temperatures, while the binding rates for all proteins are more similar at 40°C , where assembly proceeds smoothly. The temperature dependence of the protein binding rates is characterized by the Arrhenius activation energy (E_a), and there are generally two ways to explain the earlier observations in terms of activation energies. Either the activation energies for the late binding proteins are much larger than for the early binding proteins, or there is a change in rate determining step for the late proteins at low temperatures to a process with a larger activation energy.

The temperature dependence of the binding rate of each protein was measured over the accessible range (Fig. 4b), and the activation energies were determined from the slopes of the Arrhenius plots (Fig. 4b,c). The activation energies are generally quite similar for all of the proteins, scattered throughout a relatively narrow range, $\sim 24\text{--}44\text{ kcal/mol}$. The observed binding activation energies are all similar to the E_a of overall assembly determined previously, 38 kcal/mol ¹². The magnitude of the activation energies corresponds to melting of ~ 4 RNA base pairs²⁶, and also corresponds to the activation energy for folding of small proteins, although we cannot at present determine the relative contributions of RNA and protein folding to the observed kinetics. While there is a rough trend that the activation energies for the late binding proteins are somewhat larger than for the early binding proteins, the correlation is poor, and the magnitude of the differences in activation energy is insufficient to produce stalling of assembly at low temperature. Furthermore, the Arrhenius plots are linear over the accessible range (see Methods), so this clearly indicates that the activation energies do not change with temperature and thus that the rate-determining step is the same for each protein at high and low temperature.

Consequently, no one step is solely responsible for the apparent E_a of overall assembly. The slowly-binding proteins, which include both those that precede the canonical $\text{RI} \rightarrow \text{RI}^*$ transition and those that follow it, do not have the highest E_a 's (Fig. 4c), so the last steps of assembly are not more temperature-dependent than the earlier steps. Furthermore, the rates and E_a 's of the slowly-binding proteins are not well-correlated, indicating that the final stages of assembly are limited by multiple different transitions. Until now, there has been no way to follow these different transitions because the individual protein binding rates have not been determined during assembly at multiple temperatures. PC/QMS has allowed us to do this, and we find that the classic $\text{RI} \rightarrow \text{RI}^*$ mechanism is not adequate to explain the observed rates and activation energies for binding of the individual proteins.

These observations suggest that while a 21S *particle* can be isolated from assembly at low temperature, the 21S particle is not a true assembly *intermediate*. It seems likely that the reason 21S particles are retrieved from sucrose gradient purification of low-temperature assembly reactions is that a diverse collection of unstable particles that are in the process of assembling all sediment at $\sim 21\text{ S}$ until they accomplish some transition that shifts them to 26 S . This depiction agrees with the earlier observations that the characteristics of RI are variable and that

some pre-RI proteins bind only transiently at the RI stage¹³. It is likely that weakly bound proteins dissociate to different extents during the PC/QMS chase as compared to sucrose gradient centrifugation, so that the binding of some “pre-RI” proteins (particularly S5, S12, and S19) is observed to be slow by PC/QMS.

The slight clustering in protein binding rates at 15 °C (Fig. 4a,c) may indicate the presence of populated assembly intermediates. However, because the members of a group do not share the same activation energy (Fig. 4c), it appears that the binding of the proteins within a given group are not all limited by a single RNA folding step. Assembly via a variety of local transitions rather than a single, global step allows for the various subunits in a population to assemble into the native structure by a variety of routes rather than a requisite pathway. Equilibrium footprinting of reconstituted RI and RI* particles indicates that conformational changes are scattered throughout the 16S rRNA sequence, although centered on the active site¹⁴. This observation is consistent with the presence of many local conformational changes that may take place in parallel during late stages of assembly. Thus, just as macromolecular folding pathways have been expanded to folding landscapes that can be traversed by any of a variety of parallel pathways^{27,30}, so too can the assembly of a multicomponent complex, the 30S subunit, now be represented by a landscape (Fig. 5).

An assembly landscape for the 30S subunit

In the landscape representation, all possible conformations of the 16S rRNA map onto a free energy surface, but in the absence of proteins, the native 30S conformation is energetically unfavourable. Folding can proceed along many possible pathways to the native state because the landscape is composed of many local and modest barriers. A unique feature of the 30S landscape, compared to unimolecular folding landscapes²⁷, is the intermolecular protein binding, which alters the shape of the free energy surface during the assembly process. Once RNA folding produces a new binding site, protein binding creates new downhill directions by which further RNA folding can proceed. The dramatic alteration of the 16S folding landscape that accompanies ribosomal protein binding is analogous to the changes in protein folding landscapes that occur upon shifting from denaturing to native conditions. Each protein binding event further stabilizes the native 30S conformation, until all assembly pathways converge at this state. Despite the changes in the landscape that accompany protein binding, the heights of the various barriers encountered on any particular pathway appear to be quite similar.

Viewing 30S assembly as a landscape is supported not only by the detailed kinetic data reported here, but also by the classical equilibrium data summarized in the assembly map (Fig. 2b), which shows that the ribosomal proteins do not have an absolute dependence on each other for binding, but rather can bind in a variety of orders³¹. Indeed, Nomura and colleagues predicted that assembly actually proceeds by multiple pathways even as they proposed the simple RI→RI* model, because they observed that different proteins potentiated the formation of RI* particles to different extents¹³.

Assembly via a global rate-limiting step, which would be represented by a bottleneck on the landscape, could bring assembly to a standstill under non-optimal conditions. Assembly through a landscape of different barriers, on the other hand, would mean that slowing any one of the steps would slow, but not completely stall, assembly. Such a robust assembly landscape is surely one of many functions encoded by strongly conserved ribosomal sequences. RNA and protein chaperones are expected to play a role in assembly, and the protein chaperone DnaK has been specifically implicated in aiding 30S assembly^{32,34}. The landscape model developed here predicts that there are many folding transitions that are points at which chaperones might assist.

The assay introduced here, PC/QMS, has made it possible to begin constructing an assembly landscape for a large macromolecular complex, the 30S ribosomal subunit. The assay reports the kinetics at which different sites throughout the 30S subunit assemble, and it can be conducted under a variety of conditions designed to mimic the intracellular assembly reaction and using 30S components engineered to assess the roles of particular components and functional groups. We expect that themes from the 30S assembly landscape will inform our understanding of the assembly of RNPs and of large complexes in general. As a general method suitable for studying site-specific assembly in multicomponent complexes, PC/QMS can also be adapted to these systems.

METHODS

Pulse-chase assembly of 30S subunits. Mixtures of all 30S proteins (unlabeled or ^{15}N -labeled) and 16S rRNA were prepared from native 30S subunits as described in Supplementary Information. Binding titrations indicate that the concentrations of active proteins in the mixtures are approximately stoichiometric (within ~ 2 -fold), so differences in the concentrations of the proteins should have a minimal effect on the observed binding rates. Standard assembly conditions were $0.3\ \mu\text{M}$ 16S and $0.45\ \mu\text{M}$ ^{15}N -proteins in assembly buffer ($25\ \text{mM}$ Tris-HCl pH 7.5 at room temperature, $330\ \text{mM}$ KCl, $20\ \text{mM}$ MgCl_2 , $2\ \text{mM}$ DTT), $40\ ^\circ\text{C}$ ³⁵; the chase was 5x unlabeled proteins. Nonspecific binding of the excess proteins in the chase was resolved by purifying the assembled 30S subunits in 10-40% sucrose gradients containing a high salt concentration (assembly buffer with $0.5\ \text{M}$ NH_4Cl). Particles assembled under standard conditions in the presence of excess proteins and purified in high-salt conditions are properly formed, as judged by the extent to which they bind 50S subunits to form particles that migrate as 70S particles³⁶. (Assembled subunits are somewhat less active than native 30S; assembled and chased subunits are as active as those that are not chased.) The PC/QMS assay was performed at 40 , 30 , and $15\ ^\circ\text{C}$. The very low rate of assembly at low temperatures makes $15\ ^\circ\text{C}$ the lowest temperature at which it is practical to measure binding kinetics; over the course of a six-day experiment at $10\ ^\circ\text{C}$, some precipitation was observed in protein samples, causing concerns about the integrity of samples over the long periods of time required for assembly at such low temperatures (see Supplementary Table S1).

MALDI analysis. The proteins bound during the pulse-chase reaction were extracted from the assembled 30S subunits as described in Supplementary Information. The extracted proteins were analysed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems) operated in linear mode. The intensities of the protein peaks were determined by fitting each peak to a single gaussian function using Igor Pro (WaveMetrics). The heights of the gaussian fits (after background subtraction, see Supplementary Information) were taken as the peak intensities. The relative ^{15}N -protein intensities, ^{15}N -protein/ $(^{14}\text{N}$ -protein + ^{15}N -protein), are reported.

Analysis of protein binding progress curves. The progress curves of relative ^{15}N -protein intensity vs. time were fit to an equation of two-state binding of a bimolecular system, $\text{R} + \text{P} \rightarrow \text{RP}$:

$$d[\text{RP}] / dt = k_{\text{on}}[\text{R}][\text{P}] - k_{\text{off}}[\text{RP}]$$

where R is 16S rRNA, P is one of the proteins, and RP is the complex. Because ^{15}N -protein binding was chased with 5x ^{14}N -proteins, the minimum fraction of ^{15}N -protein bound was 0.17 ($1/(1+5) = 0.17$). The observed binding rate is the product of k_{on} and the total RNA concentration ($k_{\text{obs}} = k_{\text{on}}R_{\text{T}}$). For most proteins, this observed binding rate likely represents many rate constants—binding of the protein itself as well as earlier proteins and rRNA folding.

Arrhenius analysis. The activation energies of protein binding are calculated using the Arrhenius equation, $k = Ae^{-E_a/RT}$, from the slopes of the Arrhenius plots.

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Figure 1.
The PC/QMS method for measuring protein binding kinetics in the 30S ribosomal subunit. **a**, Schematic of the method. **b**, Quantification of relative ^{15}N -protein concentrations for several proteins from standard mixtures of native ^{15}N - and ^{14}N -30S subunits. The average relative intensities for all proteins from the three mixtures were 0.24 ± 0.03 , 0.50 ± 0.03 , and 0.73 ± 0.04 (errors denote s.d.). **c**, MALDI-TOF mass spectrum of 30S proteins from the 2-min timepoint of an assembly reaction performed under standard conditions. The inset shows expanded spectra for several timepoints for proteins S18 and S13. Additional details are provided in Supplementary Information.

Figure 2.
Binding kinetics for 30S proteins from PC/QMS under standard conditions. a, Representative progress curves for protein binding (see Supplementary Fig. S2), fit as described in Methods. The error bars are derived from the s.d. of standard samples (Supplementary Information). **b,** Proteins in the Nomura assembly map^{10, 11, 37} are coloured by their binding rates (Supplementary Table S1) (red: 20 - ≥ 30 min⁻¹, gold: 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). S5 is shown in green and blue to represent the binding rates of the unacetylated and acetylated forms, respectively. The grey bar represents 16S rRNA. **c,** Proteins in an X-ray crystal structure of *T. thermophilus* 30S¹ are coloured as in **b**.

Figure 3.

The ratio of the observed protein binding rates at two concentrations vs. the rates at standard concentration. Ratios of 1.0 or 0.13 (dashed lines) would indicate unimolecular or bimolecular rate-limiting steps, respectively. The errors in k_{obs} (s.d. from the fits of progress curves) are propagated to produce the errors bars. The proteins that bind very rapidly at the standard concentration are not shown, because the rates cannot be accurately determined from the present data. S10 data are not shown due to poor signal. Proteins S6 and S8 have high ratios, similar to two other central domain proteins, S18 and S15. Proteins S16, S17, and S20 have lower ratios, similar to most proteins.

Figure 4.

The temperature dependence of protein binding rates. **a**, The fits of binding progress curves at 15 °C, coloured according to the rates (Supplementary Table S1): orange: 4.4 - 21 min⁻¹; green: 1.0 min⁻¹; aqua: 0.044 - 0.11 min⁻¹; purple: 0.00096 - 0.010 min⁻¹. Post-RI* proteins (S3, S10, and S14) are shown as dashed lines here and in **b**. **b**, Arrhenius plots of the observed rates (see Supplementary Fig. S3). The error bars are from the errors in k_{obs} (s.d. from the fits of progress curves). The proteins that bind very rapidly are not shown here or in **c**. **c**, Protein binding rates at 15 °C vs. the activation energies (Supplementary Table S1). The errors in E_a are the s.d. from the linear Arrhenius plot fits. Proteins are coloured by 30S domain (magenta: 5', cyan: central, purple: 3'). Post-RI* proteins have large points.

Figure 5.

An assembly landscape for 30S assembly. The horizontal axes of the surface correspond to 16S rRNA conformational space, and the vertical axis is free energy. The native conformation of the 16S rRNA adopted in the 30S subunit is located at the bottom corner. In the absence of proteins, this is not the lowest-energy conformation of the RNA. Parallel folding pathways are indicated by the arrows on the energy surface. Local folding creates protein binding sites, and major changes in the landscape accompany protein binding (coloured spheres). Sequential protein binding eventually stabilizes the native 30S conformation. All pathways converge on this point, and there is no bottleneck through which all folding trajectories must pass.