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Physical Characteristics of the Reconstitution Intermediates (RI_{30} and RI_{30}*) from the 30S Ribosomal Subunit of *Escherichia coli*†

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ABSTRACT: The isolated reconstitution intermediates (RI_{30} and RI_{30}*) from the 30S ribosomal subunit of *Escherichia coli* were found to contain ten proteins. The sedimentation ceofficients, diffusion coefficients, density increments, extinction coefficients, and molecular weights were determined for the reconstitution particles and compared with those obtained from the 16S rRNA under identical buffer conditions. The results show that the binding of the proteins on the 16S rRNA at 4 °C does not markedly affect the folding of the RNA molecule. However, upon heating the RI_{30} particle at 40 °C to form the RI_{30}* particle, significant folding of the RNA took place, giving a structure considerably more compact than that of the 16S rRNA or the RI_{30} particle.

An active 30S ribosomal subunit from *Escherichia coli* can be reconstituted in vitro from its components, the 16S rRNA and the 21 proteins (Traub & Nomura, 1969). The assembly process has been studied extensively (Held & Nomura, 1973; Nomura, 1973), and the reaction follows the sequential steps

$$\text{16S rRNA + RI proteins} \xrightarrow{\text{0–10 °C}} RI_{30}\text{ particles} \quad (1)$$

$$RI_{30}\text{ particles} \xrightarrow{\text{heating}} RI_{30}^*\text{ particles} \quad (2)$$

$$RI_{30}^*\text{ particles + split proteins} \rightarrow \text{30S particles} \quad (3)$$

The "RI proteins" are defined as protins contained in the intermediate particle (RI_{30}), which has a sedimentation coefficient $s^0_{20,w}$ of 21.3 ± 0.7 in low-salt buffer (Traub & Nomura, 1969). The RI_{30}* particle is formed by heating the RI_{30} to 37–40 °C for 30 min. The sedimentation coefficient for the RI_{30}* particle has been reported to be 25–26 S in low-salt buffer on a sucrose gradient (Held & Nomura, 1973). These reports together with the observation by Hochkeppel & Craven (1977) strongly suggested that the heating step caused a folding in the conformation of the RI_{30} particle in order to form the RI_{30}* particle.

Recently, Dunn & Wong (1979) reported sedimentation coefficients for the RI_{30} and RI_{30}* particles to be 29.4 S and 26.5 S, respectively, and suggested that the RI_{30}* particles is more asymmetric and/or less compact than the RI_{30} particle. Their values were obtained in high-salt buffer and, in the case of the RI_{30} particle, in the presence of split proteins.

In order to better understand the molecular mechanism of the in vitro assembly process, we need to completely isolate and physically characterize the intermediates. We report here the sedimentation coefficients, diffusion coefficients, density increments, extinction coefficients, and the molecular weights of the isolated RI_{30}, RI_{30}*, and 16S rRNA in low-salt buffer. From these results it appears that the binding of the "RI proteins" on the 16S rRNA at low temperature is not responsible for the folding of the RNA molecule since the overall dimensions of the RI_{30} particle were found to be slightly larger than those of the RNA molecule itself. However, the heating step that converts the RI_{30} particle into RI_{30}* particle does cause massive folding of the RI_{30} particle. The RI_{30}* particle

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is considerably more compact than either the 16S rRNA or the RI_{30} particle.

Materials and Methods

The following buffers were used: buffer I, 0.01M Tris-HCl, pH 7.4, 0.1 M KCl, and 0.0015 M $MgCl_2$; buffer II, 0.03 M Tris-HCl, pH 7.4, 0.02 $MgCl_2$, and 6 mM β-mercaptoethanol; buffer III, buffer II with 0.5 M KCl; buffer IV, 0.005 M K_2HPO_4, pH 7.4, 0.02 M $MgCl_2$, 1 M KCl, and 6 mM β-mercaptoethanol; reconstitution buffer, 0.03 M Tris-HCl, pH 7.4, 0.02 M $MgCl_2$, 0.33 M KCl, and 0.006 M β-mercaptoethanol.

Preparation of Ribosomes. Ribosomes were prepared from *Escherichia coli* MRE 600 harvested in the middle logarithmic phase, essentially using the method of Hill et al. (1969b). In the final step, the 50S and 30S subunits were separated on a 10–30% exponential sucrose gradient formed in a Ti-15 Beckman zonal rotor spun at 32000 rpm for 14.5 h. The 30S subunits were recovered from the sucrose fractions by precipitating with 2 volumes of ethanol after the Mg^{2+} and dithiothreitol concentrations were raised to 0.01 and 0.001 M, respectively. The precipitate was pelleted by centrifugation at 20000*g* for 30 min and dissolved in and dialyzed at 4 °C against buffer I overnight. The purity of the ribosomal preparation was checked routinely by means of sedimentation velocity in the Beckman Model E analytical ultracentrifuge. The 30S subunits were used immediately for RNA and protein extraction.

Purification of 16S Ribosomal RNA and Total Proteins. The 16S rRNA was prepared from the 30S subunits by the method of Stanley & Bock (1965). After the final precipitation of RNA with 95% ethanol, the RNA was dissolved in buffer II and dialyzed 24 h with at least two changes of dialysate before the reconstitution step. RNA samples used for physical measurements were dialyzed against buffer I instead for 36 h with three changes of dialysate before any physical data were taken.

The total proteins from the 30S subunit (TP 30) were prepared by extracting the subunits with 8 M urea and 4 M LiCl according to Traub et al. (1971). The 30S ribosomal subunits at a concentration of 10 mg/mL were incubated with an equal volume of 8 M urea and 4 M LiCl for 24 h. The precipitated RNA was removed by centrifugation at 10000 rpm for 30 min. The resulting TP 30 solution was first dialyzed against buffer IV for 12 h and then against buffer III for another 24 h with two changes of dialysate.

Preparation of RI_{30}. The method of Traub et al. (1971) was used for the preparation of the intermediates. TP 30 in buffer III were mixed with 16S rRNA in buffer II at a volume and molar ratio of 2:1 at 4 °C. The RI_{30} particles formed in the reaction were separated from the unbound proteins by passing through a 30% sucrose cushion, prepared with the reconstitution buffer, in a Ti 60 rotor spun at 60000 rpm for 7.5 h. The pellet was suspended in buffer I and dialyzed against the same buffer for 6–8 h in order to dissolve the RI_{30} particle. The RI_{30} samples used for physical measurements were dialyzed against buffer I for 36 h with at least three changes of dialysate before any physical data were taken.

Preparation of RI_{30}.* The RI_{30} particle in buffer I was dialyzed against the reconstitution buffer for 24 h with several changes of dialysate to bring the RI_{30} particle into the high-salt condition. The RI_{30}* partcle was then prepared by heating the RI_{30} particle in reconstitution buffer at 40 °C for 40 min. The sample was cooled rapidly in ice and dialyzed for 12 h against buffer I before passage through a 10–30% sucrose gradient, prepared with buffer I, in a Beckman Ti 14 zonal rotor spun at 48000 rpm for 11 h. The RI_{30}* particles were recovered by ethanol precipitation and dialyzed against buffer I to equilibrium before physical measurements were taken.

Preparation of Reconstituted 30S Particle. The reconstituted 30S particles were prepared by adding TP 30 to RI_{30}* particles without heating. In a typical experiment, TP 30 extracted from 2 mg of 30S subunits were diluted to 1 mL and added to an equal volume of RI_{30}* particles at 2 mg/mL in reconstitution buffer at 0 °C. The reaction mixture was stirred in the cold for 5 min, and the activity of the reconstituted 30S particles were tested in the binding assay without further purification.

Protein Composition. The protein compositions of the RI_{30} and RI_{30}* particles were established by two-dimensional gel electrophoresis (Kaltschmidt & Wittmann, 1970) after extraction with 67% acetic acid in the presence of 0.1 M $MgCl_2$ (Hardy et al., 1969).

Poly(U)-Directed Phe-tRNA Binding Assay. The activity of the reconstitution intermediates and the 30S particles which were formed by adding proteins to the RI_{30}* particles was assayed by the poly(U)-directed Phe-tRNA binding system (Nirenberg & Leder, 1964). The [^{14}C]Phe-tRNA was prepared by charging *E. coli* B tRNA mixture with [^{14}C]-phenylalanine. A 5-mL reaction mixture containing 0.25 mM Tris-HCl, pH 7.4, 0.25 mM KCl, 0.1 mM KOH, 0.05 mM $Mg(OAc)_2$, 0.01 mM dithiothreitol, 0.01 mM ATP, 25 μCi of [^{14}C]phenylalanine, 30–35 mg of mixed tRNA, and S-100 enzymes was incubated at 30 °C for 25 min. The proteins in the mixture were precipitated out with 2.5 mL of phenol. The phenol and aqueous phases were separated by centrifugation. The tRNA in the aqueous phase were precipitated with 2 volumes of ethanol in 2% KOAc. The purified tRNAs were dissolved in H_2O and lyophilized.

The [^{14}C]Phe-tRNA can bind to active ribosomal particles in the presence of poly(U). For each 0.1-mL aliquot of reaction mixture, it contained 0.02–0.04 mg of poly(U), 0.02 mg of ribosomal particles, 15000 cpm of tRNA, 0.02 M Tris-HCl, pH 7.5, 0.15 M NH_4Cl, 0.03 M $Mg(OAc)_2$, and 0.0002 M dithiothreitol. The mixture was incubated on ice for 90 min, filtered on a nitrocellulose filter, and counted in a Beckman scintillation counter after drying.

Physical Measurements. Sedimentation velocity experiments were carried out in a Beckman Model E ultracentrifuge using a Beckman ANE rotor at 4 °C. The rotor was spun at 48000 rpm and schlieren optics were used. Plates were measured on a Nikon 6C microcomparator equipped with IKL digital micrometers. The apparent sedimentation coefficient was corrected for temperature and solvent differences to give $s^0_{20,w}$. These values obtained for a dilution series were extrapolated to infinite dilution by using a linear least-squares program to give $s^0_{20,w}$.

The density increment ($\partial\rho/\partial c$) (Casassa & Eisenberg, 1964) was obtained from a density vs. concentration plot. The density of each sample was determined by using a Paar DMA O2C digital densitometer (Kratky et al., 1973) as outlined previously by Ortega & Hill (1973). The slope of the density vs. concentration plot ($\partial\rho/\partial c$) was determined by a linear least-squares program. The apparent specific volume for the RNA was obtained by

$$\Phi^* = \frac{1}{\rho_0}\left(1 - \frac{\partial\rho}{\partial c}\right)$$

where Φ^* is the apparent specific volume and ρ_0 is the density of the buffer. The concentration was determined by measuring the absorbance of the sample at 260 nm.

Table I: Protein Composition of RI_{30} and RI_{30}* Particles

		M_r [a]
S1	–	65 000
S2	–	28 300
S3	–	28 200
S4	+	26 700
S5	+	19 600
S6	–	15 600
S7	+	22 100
S8	+	15 500
S9	+	16 200
S10	–	12 400
S11	–	15 500
S13	+	17 200
S13	+	14 900
S14	–	14 000
S15	+	12 500
S16	+	11 700
S17	+	10 900
S18	–	12 200
S19	–	13 100
S20	–	12 000
S21	–	12 200

[a] Values obtained from sodium dodecyl sulfate-gel electrophoresis (Dzionara et al., 1970).

The extinction coefficients were determined by measuring the absorbance of solutions at 260 nm and then determining the concentration of those solutions by means of dry weight measurements (Hill et al., 1969a). Sample solutions previously dialyzed to equilibrium were weighed in 25-mL tared volumetric flasks and dried in vacuo to a constant weight at 98–100 °C. The optical densities of duplicate sample solutions at 260 nm were carefully recorded on a Beckman DU 8 spectrophotometer.

The diffusion coefficient was determined by using intensity fluctuation spectroscopy (Pusey et al., 1974; Camerini-Otero et al., 1974; Koppel, 1974) from the correlation function

$$g(\tau) = e^{-DK^2\tau}$$

where $g(\tau)$ is the normalized first-order electric field correlation function, τ is the delay time, K is the magnitude of the scattering vector, and D is the translational diffusion coefficient. The correlation function was obtained by using Malvern 4300 spectrometer system equipped with a digital autocorrelator. Details of the method will be published elsewhere (D. P. Blair and W. E. Hill, unpublished results).

The diffusion coefficients of the 16S rRNA and reconstitution intermediates were obtained by banding samples on 10–30% sucrose gradients. Samples were spun in a Beckman SW 41 rotor at 40 000 rpm for 11, 9, and 6 h at 4 °C for the 16S rRNA, RI_{30}, and RI_{30}* particles, respectively. The diffusion measurements were made directly on the sample banded in the centrifuge tube.

Calculation of Hydrodynamic Properties. The frictional coefficient ratio (Tanford, 1961) f/f_{min}, can be calculated by using the sedimentation coefficient in the equation

$$f/f_{min} = \left(\frac{(4/3)^{1/3}}{6\eta(\pi N)^{2/3}}\right)\left(\frac{(1 - \Phi^*\rho_0)M^{2/3}}{\Phi^{*1/3}s}\right)$$

where M is the molecular weight of the macromolecule, η is the viscosity of the solvent, and N is Avogadro's number.

The effective hydrodynamic radii (Tanford, 1961), R_{sed}, were calculated by

$$R_{sed} = M(1 - \Phi^*\rho_0)/(6\pi\eta Ns)$$

where the symbols are the same as those defined above.

The molecular weight was determined by combining the diffusion coefficient, sedimentation coefficient, and the density increment $(\partial\rho/\partial c)$ in the Svedberg equation

$$M = \frac{sRT}{D(\partial\rho/\partial c)}$$

where R is the gas constant and T is the absolute temperature.

Table II: Poly(U)-Directed [^{14}C]Phe-tRNA Binding Activities of Ribosomal Particles

	cpm
30S	740
30S + TP 30	2245
RI_{30}	664
RI_{30}*	842
RI_{30}* + TP 30	1644

Table III: Physical Characteristics of 16S rRNA, RI_{30}, and RI_{30}* Particles in 0.01 M Tris-HCl, pH 7.4, 0.1 M KCl, and 1.5 mM $MgCl_2$

	16S RNA	RI_{30}	RI_{30}*
$s^0_{20,w}$ (S)	19.6	22.7	26.2
$D^0_{20,w}$ (cm^2/s)	1.68×10^{-7}	1.52×10^{-7}	2.0×10^{-7}
Φ*(mL/g)	0.531	0.548	0.582
$\partial\rho/\partial c$	0.466	0.449	0.415
$M_r \times 10^{-6}$	0.61	0.81	0.77
$E^{1\%}_{260}$	203	144	152
R_{sed} (Å)	127	141	107
f/f_{min}	2.51	2.50	1.90

Results

The isolated RI_{30} and RI_{30}* particles contain ten proteins as listed in Table I. The RI_{30} particle we isolated contains fewer proteins than that reported by Dunn & Wong (1979) but has all the proteins necessary for RI_{30}* formation. The discrepancy is probably due to the different methods of RNA preparation. We prepared our RNA by the phenol extraction method (Stanley & Bock, 1965), while Dunn & Wong used the acetic acid–urea extraction method (Hochkeppel et al., 1976), which has been reported to be able to bind more proteins. Since the purified RI_{30}* particle contains the same set of proteins as the RI_{30} particles, the heating step that converts the RI_{30} particle into the RI_{30}* particle apparently does not cause protein to dissociate from the 16S rRNA.

The poly(U)-directed Phe-tRNA binding activities of the RI_{30} particle, RI_{30}* particle, reconstituted 30S particle, and the washed native 30S subunit are listed in Table II. The binding data indicate that upon adding proteins to RI_{30}* particle, there is a marked increase in binding activity. These reconstituted 30S particles have higher activity than the washed native 30S subunits, but lower activity than the washed native 30S subunits which have been incubated with TP 30.

The physical characteristics of 16S rRNA and the reconstitution intermediates are listed in Table III. The 16S rRNA in buffer I is slightly unfolded when compared to the conformation of the 16S rRNA in reconstitution buffer (Tam et al., 1981). The sedimentation coefficients for the 16S rRNA in buffer I and reconstitution buffer are 19.6 ± 0.4 and 21.0 ± 0.5 S, respectively, while the diffusion coefficients are (1.68 ± 0.06) × 10^{-7} cm^2/s and (1.72 ± 0.02) × 10^{-7} cm^2/s, respectively. Although we detected a slight change in conformation between these two samples of RNA as indicated by the $s^0_{20,w}$ and $D^0_{20,w}$ values, the hydrodynamic radii for these two RNA samples are approximately the same (R_{sed} = 127 Å in buffer I and R_{sed} = 125 Å in reconstitution buffer) yet significantly larger than the 30S subunit in the same buffer (R_{sed} = 108 Å; unpublished results).

In comparison, the RI_{30} particle has a sedimentation

coefficient of 22.7 ± 0.5 S and a diffusion coefficient of (1.52 ± 0.04) × 10^{-7} cm^2/s. This sedimentation coefficient is close to the value obtained by Traub & Nomura (1969) but considerably different than the 29.4S value reported by Dunn & Wong (1979). However, Dunn & Wong made their measurements in the presence of split proteins and under ionic conditions considerably different than those used in this study. Our calculated R_{sed} for the RI_{30} particle is 141 Å, which is somewhat larger than the value obtained for the RNA itself.

It is interesting to note that the f/f_{min} values are the same for both the RI_{30} and 16S RNA. The frictional coefficient ratio (f/f_{min}) depends on two factors, solvation and asymmetry. We cannot directly compare the asymmetry of the RNA to the RI_{30} particle without a precise knowledge of the solvation terms for the RNA and the RNP particle. Nonetheless, the hydration of RNA (Bloomfield et al., 1974) is usually considered to be higher than most of the proteins (Kuntz & Kauzmann, 1974). If we assume the binding of proteins onto the RNA does not significantly increase the hydration property of the resulting RNP particle, we can tentatively conclude that the binding of the proteins onto the RNA does not markedly affect the overall conformation of the RNA.

The RI_{30}* particle in buffer I has sedimentation and diffusion coefficients of 26.2 ± 0.6 S and (2.00 ± 0.1) × 10^{-7} cm^2/s, respectively. The marked increase in these coefficients suggests that the RI_{30}* particle has a more compact conformation than either the RI_{30} particle or the 16S rRNA. The frictional coefficient ratio (f/f_{min} = 1.90) also indicates that the RI_{30}* particle is less asymmetric and/or hydrated than the RI_{30} particle or 16S rRNA. The calculated R_{sed} for the RI_{30}* particle is 107 Å, which is substantially lower than the RI_{30} particle or the 16S rRNA and quite similar to the 30S subunit itself [R_{sed} = 108 Å (unpublished results)].

The apparent partial specific volumes for the 16S rRNA, RI_{30} particle, and RI_{30}* particles are 0.531, 0.548, and 0.582 mL/g, respectively. Combining the apparent partial specific volume, sedimentation coefficient, and diffusion coefficient in the Svedberg equation, we obtained a molecular weight of (0.61 ± 0.02) × 10^6 for the 16S rRNA, (0.81 ± 0.03) × 10^6 for the RI_{30} particle, and (0.77 ± 0.03) × 10^6 for the RI_{30}* particle. From protein and RNA composition, the molecular weight we obtained is 0.78 × 10^6 for the reconstitution intermediate. For this calculation we used the molecular weights of proteins as obtained from sodium dodecyl sulfate gels rather than those obtained from sequencing since the former values may more closely approximate those actually found in solution. These results are all within experimental error, suggesting that there is no significant composition difference between the RI_{30} and the RI_{30}* particles.

Discussion

The RI_{30} particle is 25% heavier and 27% larger in volume than the 16S rRNA. The volume of the proteins account for most of the volume increase. However, the sedimentation coefficient of the RI_{30} particle should be 24–25 S if the proteins were added such as to cause a general conformational swelling of the 16S rRNA shape. Instead we report a 22.7S value, indicating that the RI_{30} particle is somewhat more extended and/or asymmetric than its 16S rRNA counterpart. This apparent extension could be caused by asymmetric placement of proteins themselves, extended conformation of the proteins themselves, RNA conformational change due to the binding of the proteins, significant hydration changes in the RI_{30} particle as compared to the 16S rRNA, or a combination of the above.

Upon heating of the RI_{30} particle, the RI_{30}* particle is produced. This particle has the same protein complement, but the physical characteristics are altered considerably. There is a 56% decrease in volume upon heating the RI_{30} particle at 40 °C for 40 min. The diffusion coefficient increases by 32% and the sedimentation coefficient by 15%. The apparent specific volume also increased significantly. All of these changes in parameters strongly suggest a major conformational tightening of the particle upon heating. The resulting hydrodynamic radius is almost identical with that of the 30S under identical buffer conditions (Hill et al., 1969a,b).

These results are in general agreement with those of Held & Nomura (1973), who observed a sedimentation change of 21–26 S during the RI_{30}–RI_{30}* transition. Hochkeppel & Craven (1977) have also reported that the RNA from the RI_{30}* has a greater mobility in gel electrophoresis than the RNA from the RI_{30} particle. However, results reported herein are in conflict with those reported by Dunn & Wong (1979), who reported a sedimentation coefficient of 29.4 S for the RI_{30} particle and 26.5 S for the RI_{30}* particle.

We have specifically checked our sample for the proteins present on both the RI_{30} and RI_{30}* particles and have found similar complements on both. Binding sites on the RNA have been identified for proteins S4, S16/17, and S20 (Mackie & Zimmermann, 1978), proteins S8 and S15 (Muller et al., 1979), and protein S7 (Muto et al., 1974; Yuki & Brimacombe, 1975). With the exception of S20, all the proteins mentioned above have been identified on our reconstitution intermediates. In the experiments to identify the binding sites for proteins, the binding steps were carried out at 40 °C (Mackie & Zimmermann, 1978; Muller et al., 1979), while we prepared our first intermediate by incubating the proteins with the RNA at 0 °C. It is probable that the binding site for S20 needs a thermal activating step before binding can occur.

We have also identified proteins S5, S9, S12, and S13 on our reconstitution intermediates. Protein S5 has been cross-linked to the RNA by UV irradiation and dye-sensitized photooxidation methods (Turchinsky et al., 1978; Zook & Fahnestock, 1978), even though the RNA sequences for the binding site of this protein has not been elucidated. Proteins S9 and S13 have also been found to cross-link to RNA by nitrogen mustard (Ulmer et al., 1978). Protein S12 is usually not considered as a binding protein, but the essential role of this protein in protein synthesis initiation has been well documented (Ozaki et al., 1969; Held et al., 1974; Heimark et al., 1976; Van Duin et al., 1975), and it has also been identified as a binding protein by Hochkeppel et al. (1976) on acetic acid–urea extracted 16S rRNA. Thus we can conclude that the proteins present on the particles we studied are those one would expect to be present.

The addition of proteins to the RNA causes some changes in the apparent specific volume and hydration that are difficult to estimate. While values for the apparent specific volume can be measured, the amount of change due to either the addition of the protein or the conformational tightening upon heating is not predictable.

A more difficult problem is that of hydration. The value of hydration for RNA itself is not well-known. Literature values vary from 0.63 to 2.3 g of H_2O/g of RNA (Bloomfield et al., 1974; Birnie et al., 1973; Kuntz et al., 1969; Sadykhova & Braginskaya, 1975). The value is certainly dependent upon ionic conditions, temperature, nucleotide complement, and measurement technique. The hydration of the ribosomal proteins themselves is much better known, with 0.3–0.4 g of H_2O/g of protein being an accepted value (Kuntz & Kauzmann, 1974), although Gulik et al. (1978) have recently re-

ported values in excess of twice that high by using small-angle X-ray scattering results. Assuredly the addition of proteins to the RNA with the resultant folding causes new interstices or invaginations where water can be immobilized. The result is that an accurate value for the hydration of the ribonucleoprotein complex cannot be given.

Without an accurate hydration value, it is difficult to assess the amount of actual conformational folding taking place in the transition from the RI_{30} to RI_{30}*. If it can be assumed that there is but little hydration change upon heating, the results indicate a massive folding and tightening of the RI_{30} particle into a structure that is quite compatible with that of the 30S itself.

A final note on ionic conditions. The results reported herein were all obtained in low-salt buffer conditions that are not suitable for reconstitution of the ribosome. While studies under reconstitution conditions would be more useful, suitable homogeneous preparations have not yet been possible in our hands. Nonetheless, the results reported herein are all obtained under identical ionic conditions and thus should be comparable to each other.

In conclusion we can say that (1) the overall dimensions of the 16S RNA in buffer I are comparable to those of the 16S RNA in reconstitution buffer, (2) the overall dimensions of the RI_{30} particle are significantly larger and/or more extended than the 30S subunit or the 16S RNA itself, (3) the binding of RI proteins onto the 16S rRNA to form a RI_{30} particle causes an apparent loosening and/or an increase in the asymmetry of the particle, and (4) upon heating, there is significant folding of the RI_{30} particle to form the RI_{30}* particle.

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