Binding of Ribosomal Proteins to 30S Preribosomal Ribonucleic Acid of *Escherichia coli*†

Nikolai Nikolaev and David Schlessinger*

ABSTRACT: Purified 30S pre-rRNA of *Escherichia coli* bound ribosomal proteins to form ribonucleoprotein particles. After incubations at 37° in appropriate buffers, three kinds of particles were observed in zonal sedimentation in sucrose density gradients: "$p_0$30S," formed with 30S ribosomal proteins (S proteins), sedimented at about 37 S; "$p_0$50S," formed with 50S proteins (L proteins) and 5S rRNA, and "$p_0$30S-50S," formed with a mixture of S proteins, L proteins, and 5S rRNA; both $p_0$50S and $p_0$30S-50S sedimented at about 46 S. The particles were recovered from sucrose gradients and their protein compositions determined by two-dimensional electrophoretic analysis. $p_0$30S and $p_0$50S contained essentially all the proteins found in published analyses of precursor particles from cells, or from *in vitro* reconstitution trials. However, they showed several unique features: $p_0$30S, unlike *in vivo* precursors or *in vitro* reconstitution intermediates contained strongly bound protein S3; and the complexes formed efficiently at 37°, but (unlike reconstitution intermediates) not at 0°. The $p_0$30S-50S particles showed additional features not seen in trials with mature or partially mature rRNA: (1) essentially all S proteins and L proteins were bound at 37°; included were a number of proteins not bound in trials with S or L proteins alone (*i.e.*, not in $p_0$30S or $p_0$50S: S2, S10, S11, S14, S18, S21, L20, L28); (2) the molar ratios of proteins were more comparable to those of ribosomes, with the binding of 10 L proteins increased up to 3.5-fold; (3) $p_0$30S-50S particles formed at 37°; higher temperatures characteristic of other reconstitution systems were not necessary. With their more extensive protein content, $p_0$30S-50S particles showed an RNA:protein ratio and protein composition comparable to ribosomes. The possibility is suggested of cooperative interactions and special RNA sites during L and S protein binding to 30S pre-rRNA.

Ribosome formation in *Escherichia coli* resembles the process in eukaryotic cells (Maden, 1971) in a number of respects. In particular, the 16S and 23S rRNA sequences are formed from a single promoter site (see review of Pace, 1973), and some proteins bind to the nascent rRNA (Mangiarotti *et al.*, 1968; Lindahl, 1973). The long rRNA transcript, called 30S pre-rRNA, is ordinarily cleaved during its synthesis (Kossman *et al.*, 1971; Kossman, 1972), and partially processed rRNA then appears in precursor particles ["p30S and p50S" (Hayes and Hayes, 1971)] that subsequently bind the rest of the ribosomal proteins.

The *in vivo* process cannot at present be reproduced *in vitro*. Using purified 30S subunit ribosomal proteins (S proteins) and 16S rRNA, Traub and Nomura (1969) reconstituted 30S ribosomes; and Nomura and Erdmann (1970) reconstituted *Bacillus stearothermophilus* 50S subunits from an analogous mixture of 50S ribosomal proteins (L proteins) and 5S and 23S rRNAs. Both reconstitutions involve intermediate particles ("R1") which contain most of the ribosomal proteins and are in some ways analogous to the p30S precursor particle formed *in vivo*. However, in contrast to the formation of R1, the process *in vivo*: (1) requires a much lower activation energy for the transition of ribosomal precursors to active ribosomes (see review of Nomura, 1973); (2) uses larger precursor RNAs that are relatively unmethylated (p16S RNA instead of 16S rRNA; p23S RNA instead of 23S RNA; Pace, 1973); (3) shows a somewhat different protein composition of precursor particles compared to R1 (Nierhaus *et al.*, 1973) and also shows a complex relation between 30S and 50S ribosome formation (Nashimoto and Nomura, 1970; Kreider and Brownstein, 1971).

The differences are emphasized by the failure of p16S RNA to yield active ribosomes in reconstitution conditions (Wireman and Sypherd, 1974).

In an attempt to extend *in vitro* studies to mimic more closely the process *in vivo*, we have made use of the mutant *E. coli* strain AB105 (Kindler *et al.*, 1973). In that strain, the 30S pre-rRNA remains intact long enough to permit its preparation in bulk (Nikolaev *et al.*, 1973; Schlessinger *et al.*, 1974). Since strain AB105 most probably makes ribosomes from intact 30S pre-rRNA *in vivo* (Nikolaev *et al.*, 1973), we were encouraged to begin to try to form ribosomes *in vitro* from this large primary product of transcription.

Materials and Methods

The basic experimental design was modified from that of Traub and Nomura (1969). Instead of 16S rRNA, 30S pre-rRNA was incubated with S proteins in reconstitution reactions. In a similar way, the same RNA was also incubated with L proteins and 5S rRNA, or with 5S rRNA and both S and L proteins. The complexes formed were isolated by zonal sedimentation in sucrose gradients, and their protein and RNA composition were analyzed by two-dimensional gel electrophoresis.

Preparation of Ribosomal Proteins and rRNAs. Labeled and unlabeled ribosomal proteins, and unlabeled 5S rRNA, were prepared from strain D10 grown in minimal salts medium supplemented with 0.4% glucose and 50 μg/ml of L-methionine (Gesteland, 1966). 30S pre-rRNA, labeled and unlabeled, and 16S rRNA were prepared from strain AB105 grown in minimal salts containing 0.4% glucose and

† From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received May 23, 1974.* This work was supported by Grant GB 23052 from the National Science Foundation. N. N. is on leave from the Institute of Biochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria.

0.8% Difco casamino acids (Nikolaev *et al.*, 1973). All cultures were grown at 30°.

L- and S-ribosomal proteins were prepared from cells labeled with 1 μCi/ml of [^{14}C]-L-amino acid (U) mixture (about 250 mCi/mmol; New England Nuclear, lot 828-021). Label was added to a 500-ml culture at an optical density at 420 nm of 0.1, and after three generations of cell growth, cells were harvested on ice. The cells (about 0.5 g) were washed and extracted by alumina grinding, and 30S and 50S ribosomal subunits were prepared by sucrose gradient centrifugation in 0.3 mM $MgCl_2$ according to Traub *et al.* (1971). Fractions from the gradients containing pure subunits were pooled and brought to 10 mM $MgCl_2$. The subunits were then collected, either by precipitation with one volume of 95% ethanol in 10 mM $MgCl_2$ at −20° or by centrifugation in 10 mM $MgCl_2$ for 12 hr at 250,000*g* in a Spinco 65 rotor. S proteins and L proteins free of RNA were extracted from 3-mg/ml suspensions of the corresponding subunits, with 66% acetic acid in the presence of 30 mM $MgCl_2$ (Voynow and Kurland, 1971). The protein solutions were dialyzed against 1000 volumes of 10 mM Tris-HCl (pH 7.8); 20 mM $MgCl_2$; 1 M KCl, 5 mM 2-mercaptoethanol, with two changes of buffer, for a total of 24 hr. The dialysis tubes containing the samples were then covered with 500 g of Carbowax 6000 (Union Carbide) for several hours, until the solutions were concentrated to 0.5–1 ml. The samples were dialyzed again against 1000 volumes of the same buffer, and then frozen. The protein prepared by this procedure reproducibly contained about 10,000 cpm/μg, with a yield of about 900 μg. Two-dimensional gel electrophoresis (see below) showed that the preparations were pure, as compared to published standards (Kaltschmidt and Wittman, 1970b).

Unlabeled 30S pre-rRNA was extracted and purified from 10 to 15 g wet weight of cells, as already described (Nikolaev *et al.*, 1974). As with the 30S pre-rRNA, so with the 16S rRNA, an initial gradient was used to obtain a partially purified sample. The fractions containing the peak 60% of 16S rRNA were then pooled and the rRNA was precipitated with ethanol and purified further in a second sucrose gradient. The purity of these and all other rRNA species was verified by gel electrophoresis (see below) compared to 4S, 16S, and 23S rRNA. Labeled 30S pre-rRNA was separately prepared from a 1-l. culture of strain AB105 pulse labeled for 2 min with [^{3}H]uridine, as already described (Nikolaev *et al.*, 1974).

5S rRNA was extracted and purified from 50S ribosomal subunits; 10 mg of purified subunits, prepared as above, was treated with phenol and 0.5% sodium dodecyl sulfate, and the RNA was precipitated with three volumes of 95% ethanol as already described (Nikolaev *et al.*, 1973). The RNA was redissolved at 4° in 1 ml of 0.1 M Tris-acetate (pH 6) containing 0.2% sodium dodecyl sulfate; 1 ml of 4 M NaCl was added with stirring. The suspension was stored 24 hr at −20°, and the precipitated 23S rRNA was then removed by centrifugation at 10,000*g* for 20 min. The 5S rRNA was precipitated once again with three volumes of 95% ethanol. The RNA was redissolved in 1 ml of distilled water, and the precipitations with NaCl and ethanol were repeated. The final pellet of 5S rRNA (30–40 μg) was dissolved in 100 μl of distilled H_2O.

Conditions for Binding of Proteins to 30S Pre-rRNA. We have tested a number of variables for their effect on protein binding to 30S pre-rRNA (see Results). However, the standard buffers and conditions were normally those used by Traub and Nomura in reconsitution trials with 16S rRNA (1969), except that incubations were at 37° instead of 42°. A typical trial was carried out in a total volume of 150 μl. First, 20 μg of 30S pre-rRNA in 100 μl of 10 mM Tris-HCl (pH 7.8)–20 mM $MgCl_2$–5 mM 2-mercaptoethanol were incubated for 2 min. Then, 50 μl of the preparation of r proteins (see above), containing 20 μg of S proteins, or 40 μg of L proteins and 1 μg of 5S rRNA, was added. In trials in which both S and L proteins were used incubation was begun with S proteins for 10 min, and L proteins were then added. The final mixture in each case was incubated for 35 min and then chilled on ice. The mixtures were cleared of faint turbidity by centrifugation for 10 min at 10,000*g*, and the clear supernatant was applied to a linear 10–30% 17-ml sucrose gradient containing 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–300 mM KCl–5 mM 2-mercaptoethanol. The gradient was centrifuged 12 hr at 25,000 rpm in the SW27 rotor of a Spinco L3-50 ultracentrifuge at 4°. The gradient analyses are shown in Results. All analyses of protein or RNA composition were carried out on the ribonucleoprotein complexes obtained from the gradients.

Reextraction of Proteins from Complexes, and Determination of Composition by Two-Dimensional Gel Electrophoresis. The gradient fractions containing complexes of S proteins, L proteins, or both, with 30S pre-rRNA were pooled; 3 mg of the appropriate subunit, or 4 mg of 50S and 2 mg of 30S subunits, was added. The mixture was adjusted to 10 mM $MgCl_2$. Ribosomes were then collected either by precipitation with one volume of 95% ethanol at −20°, or by centrifugation for 12 hr at 50,000 rpm in the Spinco 65 rotor (the two methods gave identical results). Ribosomal proteins were then extracted with acetic acid (see above). The protein solution (about 1.5 ml) was dialyzed against 1000 volumes of 0.01 N HCl containing 5 mM 2-mercaptoethanol, and then concentrated to 1 ml against Carbowax 6000 (as above). The proteins were then precipitated with five volumes of cold acetone at −20° (Welfle *et al.*, 1972), then washed with 2.5 ml of absolute ethanol and dried *in vacuo* over phosphorus pentoxide for 24 hr.

Two-dimensional electrophoretic analysis of the proteins was carried out with the buffers and gel solutions according to the design of Kaltschmidt and Wittman (1970a), with the following modifications: (1) both the first and the second dimension were run in gel slabs (rather than using a tube gel for the first dimension); and (2) as a result, the gels could be much thinner (1.5 mm instead of 5 mm). Using these two modifications, with a slab of comparable area (20 cm × 20 cm), the gels are thin enough to permit electrophoresis at room temperature with no cooling unit for up to three slabs run back to back simultaneously. Also, while the running time in the first dimension is comparable for slab or tube gel (19 hr), electrophoresis in the second dimension requires only 21 hr (at 150 V), instead of 40 hr (at 105 V). The resultant gel electropherograms of S and L proteins were indistinguishable from the standard patterns (Kaltschmidt and Wittman, 1970b).

The exact procedure was as follows. The slab gel was formed between two 22 cm × 22 cm glass plates stood on end. Strips of plexiglass 1 cm wide and 1.5 mm thick were placed along the edges at the sides and bottom to hold the plates apart. The strips were sealed with silicone grease and held in place with binder clips.

For the first dimension, the 4% acrylamide gel was formed in three steps. (1) Additional 0.5-cm strips of plexiglass, one for each sample to be applied, were inserted par-

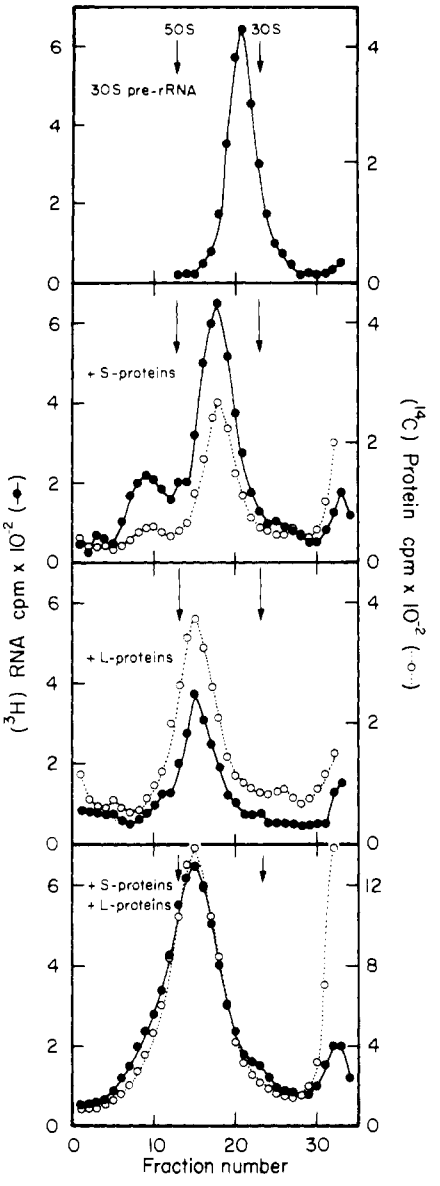

FIGURE 1: Zonal sedimentation in sucrose gradients of purified 30S pre-rRNA (top), and (below) its complexes with purified S proteins; with L proteins and 5S RNA; or with all of those components. Reaction mixtures contained ^{3}H labeled 30S pre-rRNA (16 μg containing 2100 [^{3}H]U cpm/μg) and ^{14}C protein (8000 cpm/μg, with 4.2 μg of S proteins or 8.6 μg of L proteins). Replicate trials contained twice the amounts of protein preparations with no change in the results. After incubation at 37° for 35 min in appropriate conditions (see Materials and Methods and text), samples were sedimented in 17-ml 10–30% sucrose gradients for 12 hr in the SW27 Spinco rotor at 25000 rpm. The indicated positions of authentic 30S and 50S ribosomes were determined from gradients run in parallel.

allel to the edges of the plates, to a depth of 11 cm. The gel mixture was then poured to a height of 12 cm, so that each 0.5-cm wide strip of plexiglass entered the gel to a depth of 1 cm. (In each such notch a sample is later applied.) A small amount of water was carefully applied to the gel surface between plexiglass strips, and the gel was permitted to polymerize. The water and plexiglass strips were then removed. (2) Each dry protein sample (200–300 μg of S proteins or L proteins) was then dissolved in 50 μl of sample gel, and was quantitatively applied to an individual track from a length of polyethylene tubing (1 mm diameter) attached to a 1-ml syringe. Water was again overlaid and polymerization was induced with an ultraviolet lamp (G.E. G30 T8 30-W germicidal lamp at 20 cm for about 10 min). (3) The remaining 10 cm of gel, with a composition identical with that of the lower phase, was then added, overlaid with H_2O, and polymerized. The gel was then electrophoresed in the buffer used by Kaltschmidt and Wittman (1970a).

The placement of the sample at the midpoint of the gel permitted easy resolution of the acidic and basic proteins. After a run of 19 hr at 150 V at room temperature, the glass plates were separated. Each sample track was slit out of the gel in a 0.5 × 20 cm strip, soaked in 100 volumes of dialysis buffer (Kaltschmidt and Wittman, 1970a) for 2 hr, and applied to a new gel for analysis in the second dimension.

For the second dimension, a 20 cm × 20 cm gel was prepared for each sample track from the first run. The sample track cut from the first gel was placed resting on and parallel to the bottom plexiglass strip, and the glass plates below and above it were then again clamped together. The 18% acrylamide gel solution for the second dimension was poured to the full 20 cm height, overlaid with water, and polymerized. Water and the bottom plexiglass strip were then removed, and the gel was electrophoresed 21 hr at 150 V in the buffer recommended by Kaltschmidt and Wittman (1970a).

After electrophoresis, the plates were separated. Each acrylamide slab was fixed and stained with Amido Black, and excess stain was removed according to the procedures of Kaltschmidt and Wittman (1970a). Every protein spot was identified by comparison to published patterns. Then each was cut from the gel and solubilized in 0.3 ml of protosol (New England Nuclear) according to the supplier's specifications; 10 ml of toluene-based scintillator, containing 0.5% 5-diphenyloxazole and 0.03% 1,4-bis[2-(5-phenyloxazolyl)]benzene, was added to each sample. Samples were then counted in a Packard scintillation spectrometer.

Reextraction of RNA from Complexes and Acrylamide Gel Electrophoresis. From some gradient fractions containing complexes of proteins and 30S pre-rRNA, the RNA was reextracted with phenol according to reported procedures (Nikolaev *et al.*, 1974). These RNA samples, as well as input RNA fractions, were electrophoresed 2.5 hr in 1.8% acrylamide–0.5% agarose gels containing 0.2% sodium dodecyl sulfate (see Weiss and Schlesinger, 1973; Schlessinger *et al.*, 1974).

Results

Conditions for Binding of Proteins to 30S Pre-rRNA. Figure 1 shows the analysis by zonal sedimentation of incubated 30S pre-rRNA, or of the pre-rRNA incubated with S proteins, L proteins, or both S and L proteins. The incubation conditions at 37° were as described in Materials and Methods. The pre-rRNA alone sediments in these conditions at about 33 S. The relatively sharp peaks of the RNP containing S proteins ("$p_0$30S") or L proteins ("$p_0$50S") are clear. (A variable, smaller amount of 30S pre-rRNA also sedimented at a faster rate, but with very little protein bound, in the trials with S proteins; it was not further analyzed.) $p_0$30S has an approximate sedimentation constant of about 37 S; $p_0$50S, of about 46 S. The complexes of 30S pre-rRNA and both S and L proteins are more heterodisperse, with a sedimentation rate again about 46S ("$p_0$30S–50S").

If the reaction with S proteins was incubated at 4° instead of 37°, instead of the $p_0$30S particle, only aggregates

TABLE I: Protein Composition of $p_0$30S[a] Particles.

S Protein	$p_0$30S	R1[b]	R1[c]	p30S[d]
S1	±	−	−	+
S2	−	−	−	−
S3	+	−	−	−
S4	+	+	+	+
S5	+	+	+	+
S6	−	+	−	−
S7	+	+	+	−
S8	+	+	+	+
S9	+	+	+	+
S10	−	−	−	−
S11	−	+	−	−
S12	+	+	+	−
S13	+	+	+	+
S14	−	−	−	−
S15	+	+	+	+
S16	+	+	+	+
S17	+	+	+	+
S18	−	+	−	−
S19	+	+	+	−
S20	+	+	+	+
S21	−	−	−	−

[a] Composition determined by radioactivity of corresponding protein spots in electrophoresis, as in Materials and Methods. Range of cpm for +, ∓, − as in Table III. [b] R1 composition as reviewed in Nomura (1973). [c] R1 composition from a control experiment with one of the protein preparations used to form $p_0$30S particles. [d] p30S composition from Nierhaus *et al.* (1973).

TABLE II: Protein Composition of $p_0$50S[a] Particles.

L Protein	$p_0$50S	$p_1$50S[b]	$p_2$50S[b]
L1	+	+	+
L2	+	−	−
L3	+	+	+
L4	∓	+	+
L5	+	+	+
L6	+	−	−
L7 + L12[c]	+	∓	+
L8 + L9[d]	+	+	+
L10	+	+	+
L11	+	∓	+
L13	+	+	+
L14	+	−	+
L15	+	−	+
L16	+	−	−
L17	+	+	+
L18	+	+	+
L19	+	−	+
L20	−	+	+
L21	∓	+	+
L22	+	+	+
L23	+	+	+
L24	+	+	+
L25	+	+	+
L26	∓		
L27	+	+	+
L28	−	−	−
L29	∓	+	+
L30	∓	+	+
L31	−	−	−
L32	∓	−	−
L33	−	−	+

[a] Composition determined by radioactivity of corresponding protein spots in two-dimensional electrophoresis as in Materials and Methods. Range of cpm for +, ∓, − as in Table III. [b] Composition given by Nierhaus *et al.* (1973). [c] Proteins counted together, since they are derivatives of a single protein (Möller *et al.*, 1972). [d] Proteins incompletely resolved in electrophoresis, and counted together.

of protein and low levels of protein binding to RNA were observed. Similar failure of particle formation was observed with S proteins when incubation mixtures contained 5 mM $MgCl_2$ instead of 20 mM, or 0.1 M KCl instead of 0.33 M (data not shown).

Analysis of Composition of Particles. RNA was reextracted from $p_0$30S and $p_0$50S, and was found to electrophorese as 30S pre-rRNA (data not shown); *i.e.*, no measurable breakage of the RNA occurred during binding trials.

The protein compositions of the three types of particles were analyzed by two-dimensional electrophoretic analysis as described in Materials and Methods. The results are listed for the respective particles in Tables I–III. The cpm measured on protein spots cut out of gels (Materials and Methods) are listed, corrected for the molecular weight of that protein (as in Weber, 1972). Based on those numbers, each protein is scored as present (+), absent (−), or present in low amount (±).

[In previous publications about the protein composition of RNA–protein complexes formed *in vivo* or *in vitro*, scoring was essentially restricted to +, ±, −, with no quantitation reported. We have given the actual measured numbers as well; and while the accuracy of the cpm reported is open to question (since there is no correction for possible differential quenching), they facilitate the comparisons of *relative* binding; see Tables I and II and below. Also, the cpm probably are valid as a first approximation because: (1) the yields of proteins from the complexes are nearly quantitative (Voynow and Kurland, 1971); and (2) in one trial $p_0$30S particles were centrifuged into a pellet directly from sucrose gradient fractions (with no added ribosomes). Their proteins were then analyzed in a one-dimensional tube gel according to Traub and Nomura (1969), and the intensity of stained proteins corresponded well to that obtained by the analysis of radioactive proteins.]

Table I shows the composition of $p_0$30S compared with that reported for the reconstitution intermediate formed from 16S rRNA and S proteins (Nomura, 1973) and that reported for the p30S precursor particle purified from whole cells (Nierhaus *et al.*, 1973). For comparison, column 3 shows the results we obtained in a control reconstitution experiment with 16S rRNA and our preparations of S proteins, in the conditions of Traub and Nomura (1969).

As Tables I and III indicate, the molar ratios of proteins in $p_0$30S differ by as much as sevenfold (S8 compared to S5). However, S proteins in mature 30S ribosomes are reported to show a comparable range, and proteins observed to be weakly bound in our analyses (*e.g.*, S8) correspond to "fractional" and "marginal" proteins as reported in the lit-

TABLE III: Protein Composition of $p_0$30S–50S[a] Particles.

	Proteins of $p_0$30S–50S (cpm)		$p_0$30S (cpm)			Proteins of $p_0$30S–50S (cpm)		$p_0$50S (cpm)	
S1	100	∓	200	∓	L1	1850	+	3300	+
S2	350	∓	0	−	L2	1700	+	1700	+
S3	2550	+	2800	+	L3	1250	+	900	+
S4	3750	+	3800	+	L4	750	+	240	∓
S5	4200	+	6500	+	L5	2150	+	570	+
S6	?[d]		0	−	L6	2050	+	720	+
S7	2850	+	4050	+	L7 + L12[e]	3450	+	1100	+
S8	1350	+	900	+	L8 + L9[c]	1200	+	1350	+
S9	3450	+	4300	+	L10	950	+	1200	+
S10	1050	+	0	−	L11	1350	+	1300	+
S11[b]	750	+	0	−	L13	1750	+	1700	+
S12	1050	+	1400	+	L14	1350	+	1350	+
S13	2250	+	2100	+	L15	4350	+	4450	+
S14	1650	+	0	−	L16	2650	+	1050	+
S15	2400	+	2700	+	L17	3750	+	2100	+
S16	1350	+	2400	+	L18	3350	+	1850	+
S17	1050	+	1800	+	L19	4650	+	2650	+
S18	2150	+	0	−	L20	350	∓	0	−
S19	1250	+	2600	+	L21	750	+	350	∓
S20 + L26?[c]	2000	+	4500	+	L22	2150	+	2500	+
S21	750	+	0	−	L23	750	+	950	+
					L24	2650	+	2950	+
					L25	1000	+	2700	+
					L26 + S20?[c]	2000	+	400	∓
					L27	850	+	850	+
					L28	1600	+	0	−
					L29	0	−	350	∓
					L30	250	∓	300	∓
					L31	0	−	0	−
					L32	0	−	300	∓
					L33	0	−	0	−

[a] Composition determined by radioactivity of corresponding protein spots in two-dimensional electrophoresis, as in Materials and Methods. cpm are the average of at least two independent trials, with a difference of up to ±10–15% from one trial to another (the variation was comparable, for example, to data for proteins L2, L8 + L9, L10, L11, L13, L14, L15, L22, L24, L27 in $p_0$30S–50S and $p_0$50S). cpm for gel regions containing no protein spot (about 30 cpm) have been subtracted from each value. [b] S11 spots were poorly defined; value approximate. [c] Proteins overlapped in the electrophoretic pattern, and were counted together. [d] S6 did not appear as a defined spot in the electropherogram of total ribosomal proteins. [e] Proteins counted together, since they are derivatives of a single protein (Möller *et al.*, 1972).

erature (Voynow and Kurland, 1971; Weber, 1972).

Both $p_0$30S and the control reconstitution trial lack certain proteins that are weakly present in R1 particles (Nomura, 1973). More significant is the presence in $p_0$30S of protein S3 (see Discussion).

Tables II and III give the corresponding analyses for $p_0$50S and $p_0$30S–50S. $p_0$50S contains most of the proteins found in the precursor $p_2$50S particle purified from *in vivo* (Nierhaus *et al.*, 1973). Once again, the molar ratios vary widely; but the ratios seem less extreme, and some additional proteins bind, when the complex is formed with both S and L proteins (see Discussion).

Discussion

The particles formed from 30S pre-rRNA and ribosonal proteins can be compared both to precursor particles from whole cells (Nierhaus *et al.*, 1973), and to complexes of ribosomal proteins with 16S and 23S rRNA (Mizushima and Nomura, 1970; Schaup *et al.*, 1971; Stöffler *et al.*, 1971; Zimmermann *et al.*, 1972). Quantitative comparisons of protein compositions are difficult, since previous studies have included only (+) and (−) scoring for each protein, and many proteins are present in fractional amounts in washed ribosomes and in the preparations of ribosomal proteins used (Voynow and Kurland, 1971). Qualitative comparisons are made below, and their possible significance is briefly discussed.

The Composition of $p_0$30S. Since 30S pre-rRNA contains a 16S rRNA sequence, one would expect that it could bind S proteins as 16S rRNA does. The resultant ribonucleoprotein, "$p_0$30S," formed under conditions optimal for R1, would probably have a protein composition similar to that of R1. In general this expectation is realized, and the K^+ and Mg^{2+} dependence for complex formation is comparable; but there are significant differences of $p_0$30S from R1 particles.

(1) Protein S3 is present in $p_0$30S. Protein S3 is present

in at least 0.7 equiv/30S ribosome (Voynow and Kurland, 1971; Weber, 1972), and is absolutely required for the reconstitution of 30S ribosomes with detectable activity in any of four functional assays (Nomura, 1973). However, it is present neither in R1 (Nomura, 1973), nor in p30S isolated from whole cells (Nierhaus *et al.*, 1973), nor in 30S particles formed from p16S rRNA (Wireman and Sypherd, 1974). Traub and Nomura found that it bound only when R1 was heated to effect the transition to active 30S ribosomes. Thus, the finding that S3 is present in $p_0$30S is unexpected.

S proteins (including S3) have specific binding sites on 16S rRNA, and do not bind, for example, to 23S rRNA (with the exceptions of S11 and S12; Morrison *et al.*, 1973). Therefore it is likely that S3 is bound to its normal specific site; but an alternative binding of S3 to some other site in 30S pre-rRNA has not been excluded. Further experiments are required to distinguish between these possibilities.

(2) Some proteins are missing in $p_0$30S. Proteins S6, S11, and S18, present in R1, are missing in $p_0$30S. However, these proteins are "fractional" proteins (Weber, 1972), and appear as relatively weak spots in two-dimensional electropherograms of total S proteins; and they have been reported missing from p30S particles isolated from cells (Nierhaus *et al.*, 1973). They were also missing in reconstitution trials with 16S rRNA and our protein preparations (Table I, column 3). It is possible that in the preparations we used, the concentration of these proteins was too low for adequate binding, or that they were washed off during preparative procedures.

(3) Formation of $p_0$30S shows a temperature dependence different from that reported for R1. At low temperature (4°) the 30S pre-rRNA shows no organized complex with S proteins; while in the same conditions with 16S rRNA, R1 was formed (Traub and Nomura, 1969). It is difficult to explain this discrepancy. Since 30S pre-rRNA is about four times longer than 16S rRNA (Nikolaev *et al.*, 1974), and therefore has a much more complicated structure, it is possible that the RNA will behave differently in salt solutions at 4°. As a result, in those conditions 30S pre-rRNA might be a comparatively poor substrate for ribosomal protein binding. Others have reported relatively poor binding at 0° even of some ribosomal proteins that bind directly to mature 16S and 23S rRNA (Schulte *et al.*, 1974).

The Composition of $p_0$50S. $p_0$50S contains most L proteins (Table II). It is known that most of the L proteins of *B. stearothermophilus* bind to *E. coli* 23S rRNA *in vitro* at 42° (Fahnestock *et al.*, 1973), but since no reconstitution has been achieved for *E. coli* 50S subunits a more detailed comparison of $p_0$50S to an R1 is precluded.

More can be said about the comparative analyses of precursors isolated from cells. *In vivo,* there are two reported precursors of the large subunit, $p_1$50S and $p_2$50S (Mangiarotti *et al.*, 1968; Hayes and Hayes, 1971). Protein compositions have been reported for both (Nierhaus *et al.*, 1973). Compared to them (Table II), $p_0$50S shows a number of differences, but most of the proteins are present in both $p_0$50S and $p_2$50S. As in the case of $p_0$30S, some proteins may be lost after incubations, or a different conformation of 30S pre-rRNA compared to 23S rRNA could lead to some discrepancies. However, since none of the L proteins bind to 16S rRNA (Morrison *et al.*, 1973), the binding of proteins is most probably specific.

The Composition of $p_0$30S–50S. This ribonucleoprotein, which is superficially similar to the eukaryotic "80S preribosomes" (Warner and Soiero, 1967), is the first such entity described for prokaryotes (but see below). It is not clear why the particle moves only as fast as $p_0$50S, even though it contains far more protein.

Protein analysis of $p_0$30S–50S revealed two qualitative differences from that of $p_0$30S and $p_0$50S: (1) some proteins not bound in the trials with S or L proteins alone now appeared in the complex, though often at low levels (S2, S10, S11, S14, S18, S21; L20, L28); (2) more important, the molar ratios of different proteins are much more comparable, and the levels of binding of many L proteins are higher by up to 3.5-fold (L3, L4, L5, L6, L7, L16, L17, L18, L19, L21).

Because of the more extensive binding of proteins, the $p_0$30S–50S particles contain essentially all ribosomal proteins, and show an RNA:protein ratio comparable to that of ribosomes (Figure 1).

Are the Complexes Relevant to Formation of Ribosomes In vivo? Strictly speaking these results show only that 30S pre-rRNA complexes with ribosomal proteins under appropriate conditions. Nevertheless, considering the usual specificity reported for interactions of ribosomal proteins and rRNA, it seems likely that most if not all the binding observed is specific. [In particular, Morrison *et al.* (1973) have shown that in reconstitution buffers, none of the L proteins bind to 16S RNA sequences; two S proteins do interact with 23S RNA, and this has even been suggested as *prima facie* evidence that they may exist at an interface between 30S and 50S subunits!]

Since no intact 30S pre-rRNA chains have ever been detected in wild-type cells, no particle identical with $p_0$30S–50S could occur ordinarily. Nevertheless, the 23S rRNA sequence is probably formed in part before cleavage of a nascent rRNA transcript occurs (Kossman, 1972). As a result, a ribonucleoprotein containing at least some portions destined for 30S and some for 50S ribosomes would transiently occur, and the $p_0$30S–50S particle may well be closer to such *in vivo* intermediates than are other *in vitro* constructs to date. It is encouraging that the 30S pre-rRNA can be recovered from growing AB105 cells in a particle with very similar sedimentation properties (Glazier, 1974).

Until recently the steps in ribosome biosynthesis were often inferred to be identical with those observed during the formation of R1. However, a number of differences have become clear. For example, as mentioned in the Introduction, formation of active ribosomes from R1 particles shows a high activation energy, while the process in *E. coli* does not show a corresponding temperature dependence (Michaels, 1971; Mangiarotti *et al.*, 1974, and personal communication). It is believed that heating is required for conformational changes in R1 *in vitro,* and, that instead, in the cell the same changes can occur more easily (Nomura, 1973).

Perhaps the properties of 30S pre-rRNA and its complexes with ribosomal proteins offer a hint to the mechanism *in vivo.* Particularly suggestive are the results which show strong binding of protein S3 even at 37°, and more effective binding of S and L proteins added together, compared to either group added separately (Table III). A conformation stabilized in 30S pre-rRNA may be favorable for the critical binding of certain proteins; or the binding of proteins to 16S and 23S rRNA sequences could interact cooperatively. Such effects could permit the formation of ribosomes with a lower activation energy than is observed in trials with separated, mature rRNA species and L and S

proteins. But without further supportive experiments, this notion is highly tentative. The most critical test is likely to be to cleave 30S pre-rRNA with RNase III (see Schlessinger *et al.,* 1974) before and after its interaction with ribosomal proteins, and to determine the protein composition of the fragmented complexes.

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