THE SEQUENTIAL NATURE OF *IN VITRO* FORMATION OF 23 S RNA–50 S PROTEIN COMPLEXES OF *ESCHERICHIA COLI*: DEPENDENCE ON TEMPERATURE AND MAGNESIUM

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1. Introduction

The reconstitution of functional *E. coli* 30 S ribosomal subunits from their separated components, i.e., 16 S RNA and purified 30 S proteins, demonstrated by Nomura et al. [1], showed that assembly depends solely upon molecular components and that it is a powerful tool for studying the structure and function of this subunit (see ref. [2]). Reconstitution of functional *E. coli* 50 S subunits has proven more difficult, possibly because the components of this subunit or assembled intermediates might be unstable at higher temperatures which may be necessary for successful reconstitution. Nomura et al. reconstituted 50 S subunits of *Bacillus stearothermophilus* that were active in polypeptide synthesis, from dissociated RNA and protein fractions [3]. Presumably, success was dependent, in part, upon heat stability of these components.

The report that functional *E.* coli 50 S subunits could be reconstructed from 50 S proteins, 5 and 23 S RNA's [4] could not be verified in this or other laboratories [2, 5]. We have reported, however, the sequential binding of 50 S proteins to *E. coli* 23 S RNA by temperature-dependent steps to form 28, 32, 43 and 48 S particles [5]. In this paper, proteins associated with these particles are characterized by 2-dimensional polyacrylamide gel electrophoresis and the effect of Mg^{2+} on the sedimentation of particles is examined.

2. Materials and methods

Growth of *E. coli* MRE 600 (RNAase 1⁻) and preparation of ribosomes, their subunits and molecular components have been described previously [5]. Radioactive ribosomes were prepared from cells grown in ^{14}C-labelled amino acids (10 μCi/ml) casamino acid concentration in the medium being reduced from 0.2–0.02%.

Buffer containing 0.03 M Tris-HCl, pH 7.4, 0.02 M Mg acetate, 0.3 M KCl and 6.0 mM β-mercaptoethanol [3] was used as binding buffer. Ribosomal RNA and 50 S proteins were mixed in binding buffer such that 1.2 equivalents of protein were added to each equivalent of 23 S RNA. The final concentration of ribosomal RNA was 3.5–4.0 A_{260} units/ml. After appropriate incubation, the alcohol-precipitated material was collected by centrifugation. Reconstituted particles were separated from 16 S RNA by 5–20% sucrose gradient centrifugation in 0.01 M Tris-HCl, pH 7.8, 0.05 M KCl and 1.0 mM or 10.0 mM magnesium acetate in a Spinco 25.2 rotor. S-values of particles were estimated in the same buffer by analytical sucrose gradient centrifugation with ^{14}C-labelled ribosomal subunits as internal markers using the method of O'Brien [6].

Proteins bound to isolated particles were extracted as described previously [5] and analyzed by 2-dimensional electrophoresis as described by Kaltschmidt and Wittmann [7]. In the first dimension, electrophoresis was conducted at 100 V for 20 hr and in the second dimension, at 235 V for 20 hr with a Model 490 vertical gel electrophoresis cell (E-C Apparatus Corp.).

3. Results and discussion

Binding of 50 S proteins to 23 S RNA was carried out by sequential incubation at 0,37 and 55°C and

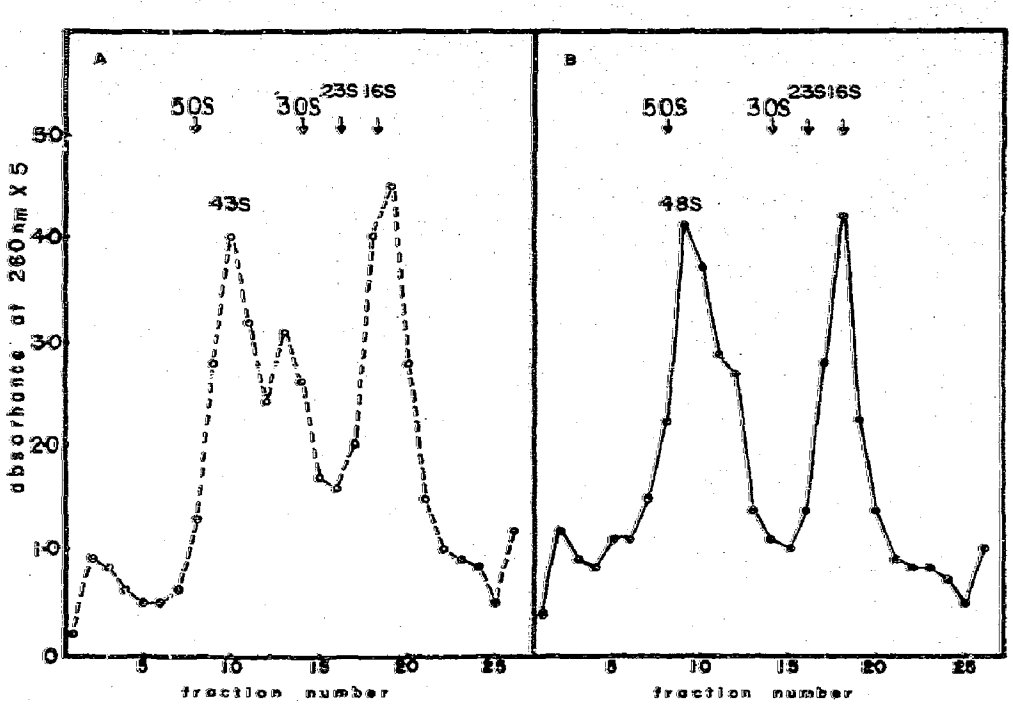


Fig. 1. Sedimentation analysis of 23 S RNA–50 S protein complexes. 12 A_{260} units of ribosomal RNA and 9 A_{260} equivalents of 50 S proteins were incubated in 3.5 ml binding buffer sequentially at 0°C for 15 min, 37°C for 15 min, and 55°C for 20 min. The alcohol-precipitated material was dissolved in 0.01 M Tris, pH 7.8, 0.05 M KCl, and 1.0 mM or 10.0 mM MgAc, then centrifuged in 5–20% sucrose gradients in the same buffer containing 1.0 mM MgAc (A) or 10.0 mM MgAc (B) in a Spinco SW 50.1 rotor for 2.5 hr at 40 000 rpm, with ^{14}C-labelled ribosomal subunits and RNA as internal markers. Fractions (0.2 ml) were collected, assayed for absorbance at 260 nm, and counted by liquid scintillation method. The arrows indicate the positions of sedimentation of internal markers.

the alcohol-precipitated material was analyzed by sucrose gradient centrifugation in low (1.0 mM) and high (10.0 mM) Mg^{2+} buffer. The results (fig. 1) show that the largest particles formed sedimented at 48 S in the 10.0 mM Mg^{2+} gradient but only at 43 S in the low Mg^{2+} gradient. The variability in formation of 48 S particles previously noted [5] may be due to low Mg^{2+} concentrations used in the analysis system. To determine if all complexes were similarly affected, 23 S RNA–50 S protein complexes were prepared at various temperatures, then isolated and analyzed in 1.0 mM or 10.0 mM Mg^{2+} buffers (Materials and methods and legend to fig. 2). The results (fig. 2) show that particles isolated and analyzed in 10.0 mM Mg^{2+} sedimented faster than those in 1.0 mM Mg^{2+}. S-values of particles produced at 0,37 and 55°C increased from 28–33 S, 32–37 S, and 43–48 S, respectively, in high magnesium concentration.

Proteins associated with each of the particles isolated in low and high Mg^{2+} buffers were analyzed by 2-dimensional electrophoresis. The results for the 28, 32, 43 and 48 S particles are shown in fig. 3. The protein patterns and staining intensity of individual proteins of the 28 and 23 S complexes formed at 0°C were identical to each other, as were those of the 32 and 37 S particles formed at 37°C, and 43 and 48 S particles formed at 55°C. Only the protein patterns of the 43 and 48 S particles are compared in fig. 3. The results indicate that increases in S-values of particles in 10.0 mM Mg^{2+} were probably due to changes in hydrodynamic properties of particles resulting from magnesium induced conformational changes, rather than to increased binding of protein. This point is important since in the binding of 30 S proteins to 16 S RNA, use of low Mg^{2+} concentrations, under similar conditions, leads to nonspecific binding of proteins

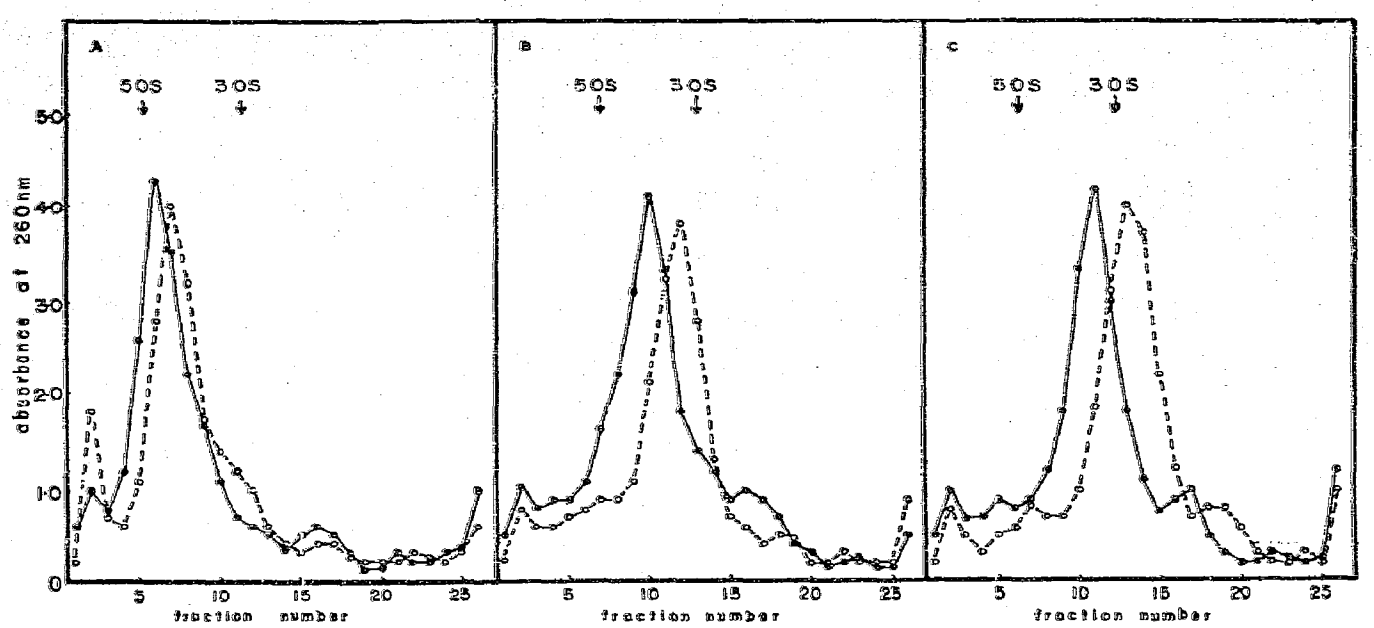


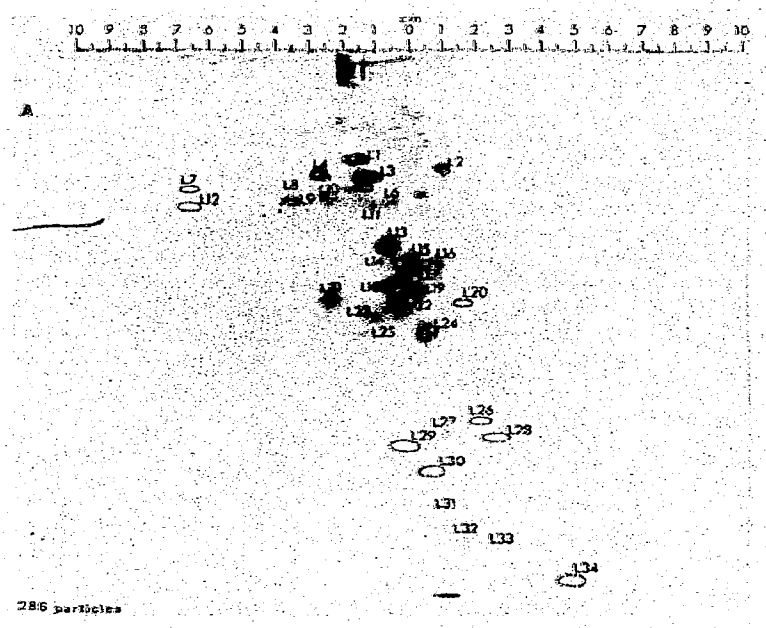
Fig. 2. Sedimentation analysis of the largest isolated particles formed: A) after 15 min at 0°C, 15 min at 37°C and 20 min at 55°C; B) after 15 min at 0°C and 15 min at 37°C and C) after 15 min at 0°C. Three A_{260} units of the isolated particles were centrifuged in 5–20% sucrose gradients in 0.01 M Tris, pH 7.8, 0.05 M KCl, and 1.0 mM MgAc (- - - -) or 10.0 mM MgAc (——) in a Spinco SW 50.1 rotor for 105 min at 50 000 rpm, analysis as described in legend to fig. 1.

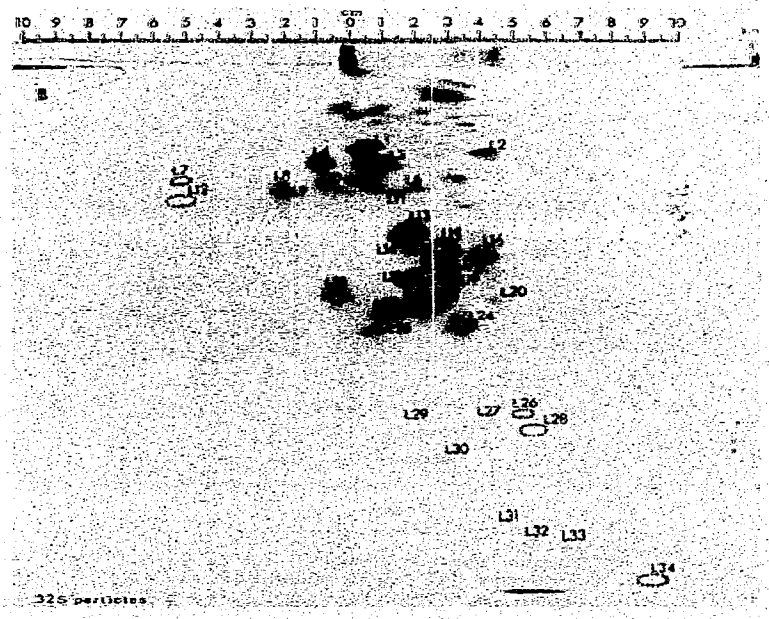
[8]. Such nonspecific binding was, therefore, not implicated in this system.

More important was the fact that definitive identification of proteins associated with each particle was possible. Since the particles formed in different Mg^{2+} concentrations at a given temperature appeared to differ only in conformation, they were identified by S-values determined in 1.0 mM Mg^{2+}, i.e., 28, 32 and 43 S in keeping with previously published data [5]. The results summarized in table 1 identify the proteins associated with each complex according to the nomenclature of Kaltschmidt and Wittmann [9]. The relative amounts of proteins were estimated by comparing the staining intensity of proteins spots with that of proteins extracted from an equivalent amount of 50 S subunits and were scored as described in footnote to table 1. The proteins could be divided into 3 major groups according to their absence or presence, and to the relative amounts present in the 3 species of particles compared to that in 50 S subunits.

The results show that binding took place sequentially so that more proteins were bound to complexes with greater S-values, and that proteins binding at higher temperatures apparently did not displace previously bound proteins. A large number of proteins were bound to 23 S RNA at 0°C in forming the 28 S particles of these proteins, 15 (group 1a) were bound in equivalent amounts, 4 (group 2a) appeared in reduced amounts in the 28 S complex, and 6 (group 1b) in reduced amounts in the 28 S, as well as 32 and 43 S complexes. The formation of 32 S particles was associated with binding of proteins that were absent in the 28 S species, and with increased binding of proteins already present in the 28 S particle. The formation of 43 S particles was related to the 32 S complexes in a similar manner by the group 3 proteins.

The largest reconstituted particles had a protein complement very close to that of 50 S subunits except 3 proteins (group 1c) were missing and 6 proteins (group 1b) were present in reduced amounts. It is interesting to note that L7 and L12 which are related to each other by reversible acetylation [10], and which have been implicated in translocation [11], are both missing from the 43 S particles. The significance of their absence is unknown, but this may be one reason


A
2816 particles


B
325 particles

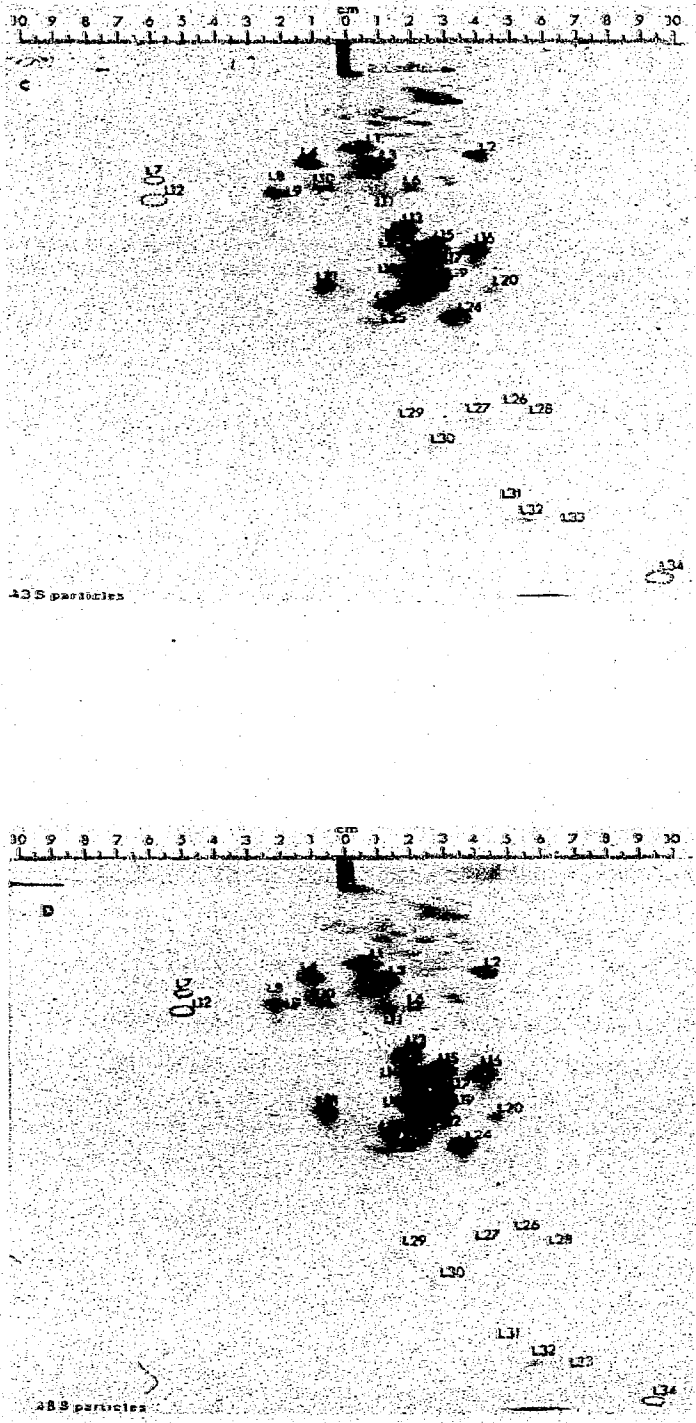


Fig. 3. Two-dimensional electropherogram of proteins of reconstituted particles: (A) 28 S particles, (B) 32 S particles, (C) 43 S particles and (D) 48 S particles. Proteins were extracted from 40 A_{260} units of the particles in 67% acetic acid in 80 mM magnesium acetate [14]. Proteins were lyophilized and dissolved in the sample gel and analyzed by two-dimensional polyacrylamide gel electrophoresis using pH 8.7 in the first dimension and pH 4.6 in the second [7]. Dotted circles represent positions of missing 50 S proteins.

Table 1
Classification of proteins in 28, 32 and 43 S particles.

Group 1 Same appearance in all particles.	
(a) Equivalent**	–L1, L2, L3, L4, L9, L13, L15, L16, L18, L19, L21, L22, L23, L24, L32
(b) Reduced***	–L5, L6, L8, L10, L11, L25
(c) Absent	–L7, L12, L34
Group 2 Initial or 'equivalent' appearance in 32 S particles.	
(a) Reduced in 28 S, equivalent in 32 S particles	–L17, L27, L31, L33
(b) Absent in 28 S, present in 32 S particles	–L30, L20*, L29*
Group 3 Initial or 'equivalent' appearance in 43 S particles.	
(a) Reduced in 32 S, equivalent in 43 S particles	–L14, L20*, L29*
(b) Absent in 32 S, equivalent in 43 S particles	–L26, L28

* Proteins are placed in group 2b and 3a.
** Equivalent, i.e., in amounts comparable to that in 50 S subunits.
*** Reduced in comparison to that in 50 S subunits.

for inactivity of these particles in polypeptide synthesis.

Stöffler et al. [12] showed that 8 proteins (L2, L6, L16, L17, L19, L20, L23, L29) could bind independently to 23 S RNA, and that equivalent amounts were bound except in the case of L6 and L17 which were present in reduced amounts. Not surprisingly, with the exception of L20 which binds to the 32 S species, all these were in the 28 S particles. L6 and L7 were found in reduced amounts in the 28 S complexes but, whereas L17 was found in equivalent amounts in the 32 S particles, the amount of L6 did not increase in the larger particles. In analogy with 30 S subunit reassembly [13], the data suggests that most of the proteins in the 28 S particles bind cooperatively, i.e., in the presence of previously bound proteins.

Pichon et al. [14] classified the 50 S proteins into 5 groups according to their *in vivo* order of addition during biogenesis of ribosomes. Of the 17 proteins that add early in biogenesis, (groups 1 and 2 of Pichon et al. [14]), 13 were bound in equivalent amounts and 2 in reduced amounts to the 28 S particles, and 2 were bound in equivalent amounts to the 32 S complexes. Two excpetions are L1 and L9 which were present in equivalent amounts in the 28 S particles but were classified as late additions in biogenesis. Proteins of group 2 and 3 that were bound in equivalent amounts to only 32 or 43 S species were late additions or missing in the Pichon analysis. The general correlation suggests that the *in vitro* binding of proteins to 23 S RNA parallels to a certain extent the *in vivo* acquisition of proteins.

The arguments supporting the contention that *in vitro* formation of 23 S RNA–50 S protein complexes represents a specific binding process have been presented before [5]. One disturbing feature observed, however, was the existence of a class of 6 proteins (group 1b) which were bound in reduced amounts to all species of particles. This indicated that the particles may be heterogeneous and that some nonspecific binding of proteins may occur which could, in part, contribute to failure in complete reconstitution. It is hoped that a more quantitative analysis presently being conducted will clarify the status of this group of proteins.

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