Ribosomal Protein S1 and Polypeptide Chain Initiation in Bacteria

(30S subunit subspecies/gel electrophoresis/initiation complex formation)

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ABSTRACT Among several subspecies of 30S subunits of *Escherichia coli* observed by polyacrylamide-agarose gel electrophoresis, only the slow-moving, protein S1-containing subspecies participates in the formation of the 30S initiation complex with coliphage MS2 RNA as mRNA; the other subspecies retain activity with AUG as mRNA; they are also active in the poly(U)-directed binding of Phe-tRNA. Protein S1 from *Caulobacter crescentus* substitutes for *E. coli* S1 despite the fact that *C. crescentus* ribosomes do not bind MS2 RNA. Under appropriate conditions, the entire population of *E. coli* 30S subunits can be isolated as the S1-containing subspecies. Protein S1 is lost by salt treatment of ribosomes.

Initiation of protein synthesis in bacterial systems involves the binding of the 30S ribosomal subunit to specific regions of mRNA accompanied by binding of fMet-tRNA (for review, see ref. 1). Electrophoresis of ribosomes on agarose-polyacrylamide gels (2) has demonstrated that, depending on the method of isolation and dissociation of the ribosomes, 30S subunit preparations contain at least two major subspecies. The main difference between the two subspecies is the presence of ribosomal protein S1 in the more slowly migrating form (3, 4). It was previously shown (4) that 30S subunits containing protein S1 form a weak, species-specific complex with MS2 RNA in the absence of initiation factors. Moreover, interference factor ia (5–7), a protein indistinguishable from protein S1 (8, 9), restores the capacity of S1-deficient *E. coli* ribosomes to bind MS2 RNA, simultaneously converting the faster to the slower 30S species (10).

In this communication we report on the further functional analysis of subspecies of 30S subunits. We find that only the S1-containing species participates in the formation of the 30S initiation complex with MS2 RNA as messenger. Both subspecies are active in the AUG-dependent ribosomal binding of fMet-tRNA and in the poly(U)-dependent binding of Phe-tRNA. We further show that protein S1 from *Caulobacter crescentus* substitutes for *E. coli* S1 despite the fact that *C. crescentus* 30S ribosomes do not bind coliphage RNA (11, 12). The subspecies composition of 30S subunit preparations obtained under various conditions is also described.

MATERIALS AND METHODS

Ribosomes of *E. coli* Q13 and MRE 600 were prepared as described (4, 12). Conditions for the isolation of 30S subunits and for assays of their activity are given in the legends to

Abbreviation: IF, initiation factor.

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figures and tables. Before use, the subunits were activated by warming for 5 min at 37° (13).

Purification of *E. coli* protein S1 from the ribosomal wash was recently described (10). Nearly homogeneous S1 was also obtained from ribosomes by the procedure of Tal *et al.* (14), followed by chromatography on agarose. The two preparations were indistinguishable by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. *C. crescentus* S1 was isolated in the course of purification of *C. crescentus* 30S ribosomal proteins in Dr. H. G. Wittmann's laboratory (S. Leffler, unpublished). Briefly, the 1.5 M LiCl split protein fraction from 30S subunits (obtained from ribosomes washed with 1.0 M NH_4Cl) was fractionated on Sephadex G-100; the first protein peak contained S1 better than 70% pure, as judged by dodecyl sulfate/polyacrylamide gel electrophoresis (15). Purified initiation factor IF2 (16) was kindly provided by Dr. R. Mazumder and Miss J. Chu. Purified IF1 (step 6, ref. 17) and IF3 (step 4, ref. 18) were used initially. Since the activity of the pure IF3 preparation decreased on storage, the 55–70% $(NH_4)_2SO_4$ fraction of the high-salt ribosomal wash was used as a source of IF1 + IF3. In agreement with others (7), we find that if the high-salt ribosomal wash is treated stepwise with ammonium sulfate at 0–35%, 35–45%, and 45–55% saturation, the subsequent 55–70% fraction will not contain S1. S1 is detected by incubation with 30S subunits (conditions as in Fig. 1), electrophoresis on polyacrylamide-agarose gels, and comparison of the UV profile of the gel with a control 30S gel profile; any shift of material from the fast band (Fig. 1A, slice 45) to the slow one (Fig. 1A, slice 41) indicates the presence of S1. Details of the polyacrylamide-agarose gel electrophoresis have been described (4, 10).

RESULTS

Agarose-Polyacrylamide Gel Electrophoresis of the 30S Initiation Complex. As shown previously (4), 30S ribosomal subunits prepared from 70S ribosomes washed with 1.0 M NH_4Cl separate into two major components which migrate faster than MS2 RNA. The gel electrophoresis method has now been applied to the analysis of the 30S initiation complex; the results of a typical experiment are presented in Fig. 1. In the absence of MS2 RNA (Fig. 1A) there is very little binding of f[^{35}S]Met-tRNA (slices 33–34). The absorbance profile of the 30S preparation alone (not shown) was identical to that in Fig. 1A. In the presence of MS2 RNA (Fig. 1C) there is a new band that moves more slowly than that of MS2 RNA (compare Fig. 1B) and coincides with a peak of f[^{35}S]Met-tRNA (slices 24–28). A shift of UV-absorbing material from the MS2 RNA band and from the slow (S1-
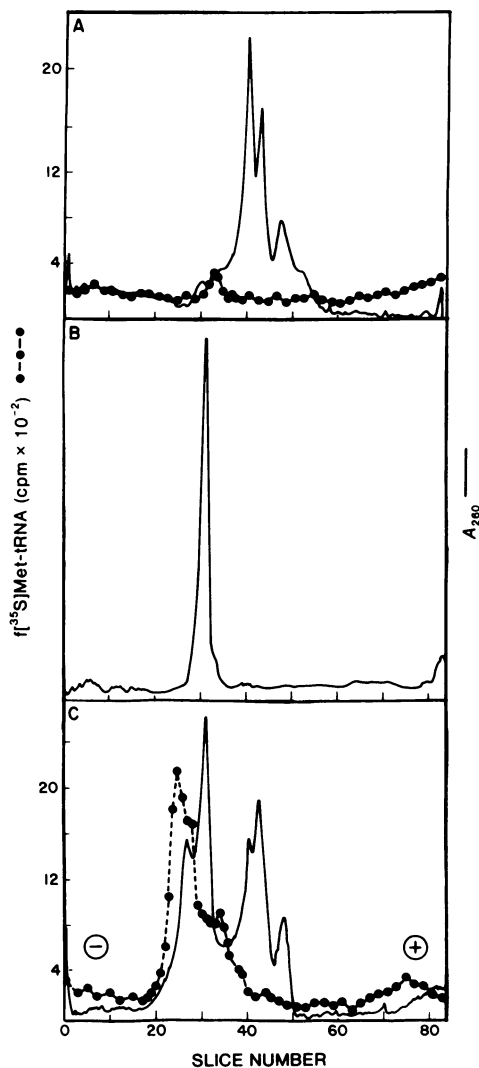
FIG. 1. Polyacrylamide (2.25%)-agarose (0.5%) gel electrophoresis of the 30S initiation complex. (A) Without MS2 RNA; (B) MS2 RNA alone; (C) with MS2 RNA. Samples (0.05 ml) contained 25 mM Tris-acetate, pH 7.4; 50 mM ammonium acetate; 5.0 mM magnesium acetate; 0.2 mM GTP; 0.5 mM dithiothreitol, 0.45 A_{260} unit of *E. coli* Q13 high-salt washed 30S (previously activated by warming for 5 min at 37° at 20 mM Mg^{2+}); f[^{35}S]Met-tRNA (2 × 10^5 cpm); IF1 + IF3, 8 µg; IF2, 2.0 µg; MS2 RNA, 0.26 A_{260} unit, where indicated. Incubation was for 5 min at 37°. Samples were layered on gels (0.6 × 9.0 cm), electrophoresed, and analyzed as described (4, 10). Complex formation was determined simultaneously by Millipore filtration; 46,740 cpm of f[^{35}S]Met-tRNA were bound with, and 3890 cpm without, MS2 RNA.

containing) 30S band to the new slower species is clearly seen. This identifies the band in slices 24–28 as the 30S initiation complex. The fast 30S band (peak slice 45) and another fast minor band (slice 49) do not seem to participate in complex formation. Some degradation of the complex occurred, probably during electrophoresis (approximately 4.5 hr at 4–8°), as judged by the trailing radioactivity in slices 32–43. Virtually identical results were obtained when the 30S initiation complex was formed and electrophoresed at a higher Mg^{2+} concentration (10 mM), except for an increased radioactive peak in the absence of added MS2 RNA (slices 33–34).
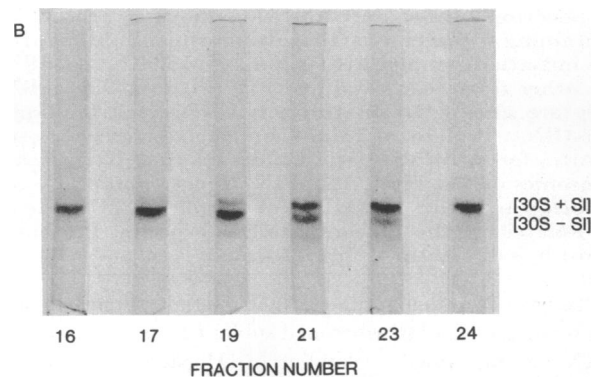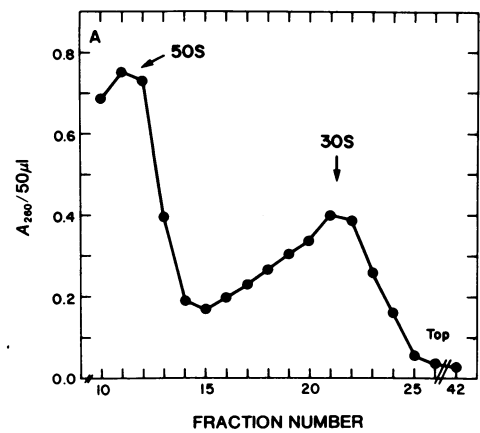

FIG. 2. Analysis of the 30S region of the sucrose density gradient by gel electrophoresis. 300 A_{260} units of *E. coli* Q13 ribosomes [pelleted from preincubated S-30 extracts (12), and repelleted from a buffer containing 20 mM Tris·HCl, pH 7.8/ 10 mM Mg^{2+}/50 mM NH$_4$Cl/0.2 mM dithiothreitol] were layered on 54 ml of a 10–30% sucrose density gradient containing 10 mM Tris·HCl, pH 7.4/0.05 mM $MgCl_2$/0.6 mM NH$_4$Cl/0.1 mM dithiothreitol, and centrifuged for 16 hr at 21,000 rpm in the Spinco SW 25.2 rotor at 4°. (A) Absorbance profile of the 30S region. (B) Gel electrophoresis (Fig. 1 and ref. 4) of selected fractions (0.3–0.4 A_{260} unit, 50–80 µl).

Attempts to analyze an initiation complex with the trinucleotide diphosphate ApUpG (AUG) instead of MS2 RNA as messenger failed; apparently the complex did not withstand electrophoresis under the conditions used.

Fractionation of 30S Subunits by Sucrose Density Gradient Centrifugation. In order to analyze their activity in various partial reactions, we separated 30S subspecies by sucrose density gradient centrifugation (3, 19). Each fraction of the 30S region of the gradient (Fig. 2) was electrophoresed as in Fig. 1; the gels were scanned at 260 nm, and the amount of subspecies was determined by quantitation of the scans. As seen in Fig. 2, the heavier S1-containing species [30S + S1] sediments somewhat more slowly than the species that does not contain S1 [30S − S1]. Each fraction of the 30S region was assayed for activity in 30S initiation complex formation (Millipore assay) with MS2 RNA and AUG as messengers. Table 1 shows typical results with two selected fractions of the gradient (see Fig. 2), both containing nearly identical overall concentrations of 30S but different amounts of each subspecies. Activity with MS2 RNA depends on the content of the [30S + S1] species in a given fraction (see preceding section), whereas activity with AUG parallels the

TABLE 1. *Activity of subspecies of 30S subunits (assay of sucrose gradient fractions of Fig. 2)*

1. Fraction number	18	23
2. 30S content/sample (A_{260} units)	0.265 (23 pmol)	0.255 (22 pmol)
3. Content of [30S + S1] (%)[a]	10 ± 3	70 ± 5
4. f[35S]Met-tRNA bound (cpm)[b]		
a. with MS2 RNA	14,260 (3,120)	58,560 (2,780)
b. with AUG	84,360 (4,650)	71,850 (3,280)
5. Poly(U)-directed binding of [3H]Phe-tRNA (cpm)[c]		
a. at 5 mM Mg^{2+}	6,360 (460)	10,240 (380)
b. at 10 mM Mg^{2+}	15,620 (1,105)	19,450 (1,260)
c. at 20 mM Mg^{2+}	27,450 (1,820)	25,830 (1,670)
6. Binding of [14C]poly(U) (cpm)[d]		
a. at 5 mM Mg^{2+}	330 (65)	860 (105)
b. at 10 mM Mg^{2+}	380 (95)	1,020 (160)

All assays were done by Millipore filtration. Figures in parentheses are blanks without mRNA.

[a] Determined by scanning gels of Fig. 2.

[b] Samples (0.05 ml of each fraction) were made 20 mM in Mg^{2+}, warmed for 5 min at 37° (13), cooled, and supplemented with 0.05 ml of fraction 6 (50S, see Fig. 2) and with components of the binding reaction given in Fig. 1 (or with 0.1 A_{260} unit of AUG/sample instead of MS2 RNA) to a final volume of 0.2 ml. Incubation was for 5 min at 37° with MS2 RNA and for 15 min at 24° with AUG. The f[35S]Met-tRNA used in this experiment had a specific radioactivity of 6000 ± 600 cpm/pmol.

[c] Samples (final volume 0.2 ml, ionic composition as in Fig. 1) were processed as in footnote b and supplemented with [3H]-Phe-tRNA (10^5 cpm) and with 10 μg of poly(U). Incubation was for 25 min at 24°.

[d] Samples were supplemented with [14C]poly(U) (12,300 cpm) only and incubated for 10 min at 10°.

overall 30S absorbance profile. Similar results were obtained in three other experiments (two with *E. coli* Q13 30S and one with *E. coli* MRE 600 30S).

Each fraction of the gradient (Fig. 2) was also assayed for poly(U)-dependent binding of [14C]Phe-tRNA. As seen in Table 1, experiment 5, this binding occurs irrespective of the presence or absence of S1 at concentrations of Mg^{2+} greater than 5 mM. When poly(U)/Phe-tRNA reaction mixtures are subjected to gel electrophoresis as in the legend of Fig. 1, it is found that, at [Mg^{2+}] above 5 mM, the [30S − S1] species is bound to poly(U) §. Under these conditions the minor fast species (Fig. 1, slice 49) is hardly affected. The binding of [3H]poly(U) to the 30S subunits (Table 1) is more dependent on the presence of [30S + S1] than the poly(U)-directed binding of Phe-tRNA. Phe-tRNA is known to enhance ribosomal binding of poly(U) (20).

§ Absorbance profiles of gels with poly(U)-30S-Phe-tRNA complexes depend on the molecular weight and on the concentration of the poly(U) preparation. At a low concentration of poly(U) of relatively high molecular weight ($s_{20,w} > 4$), large 30S-poly(U) polysomes are formed which do not enter the gel. With an excess of the polymer ($s_{20,w} < 4$), poly(U)-30S mono-, di-, and oligosomes could be seen on the gel.

[30S + S1]
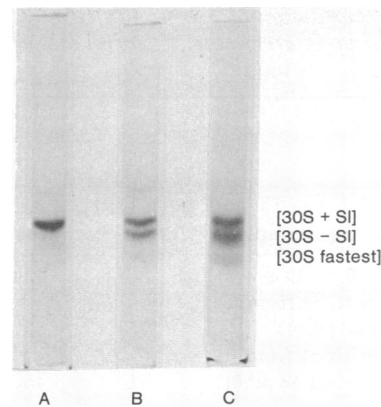[30S − S1]
[30S fastest]

A   B   C

FIG. 3. Subspecies composition of 30S subunit preparations isolated under various ionic conditions. Gradients (10–30% sucrose) were prepared, centrifuged, and analyzed as in the legend of Fig. 2. Samples for gel electrophoresis (pooled peak fractions of the 30S region) contained 0.3–0.5 A_{260} unit of 30S. (A) Ribosomes (from a preincubated S-30 extract, ref. 12) were washed in low-salt buffer (10 mM Tris·HCl, pH 7.8/10 mM Mg^{2+}/50 mM NH_4Cl/0.1 mM dithiothreitol); gradient buffer was 1.0 mM in Mg^{2+} and 50 mM in NH_4Cl; (B) as in (A), except that gradient buffer was 0.05 mM in Mg^{2+} and 0.6 mM in NH_4Cl; (C) preincubated ribosomes were washed in high-salt buffer (1.0 M in NH_4Cl) for 6 hr at 0°; gradient buffer was 1.0 mM in Mg^{2+} and 100 mM in NH_4Cl.

Effect of Washing on Relative Proportions of 30S Subspecies. As analyzed by polyacrylamide agarose gel electrophoresis, the 30S region of low-salt-washed ribosomes, sedimented through a sucrose density gradient in 1.0 mM Mg^{2+} and 50 mM NH_4^+, consists predominantly of the slow moving, S1-containing 30S subspecies (Fig. 3A). Decrease of the Mg^{2+} and NH_4^+ concentrations in the gradient to 0.05 and 0.6 mM, respectively, increases the fast-moving at the expense of the slow-moving component (Fig. 3B), i.e., leads to a release of S1. When the ribosomes are washed in high salt, with 1.0 mM Mg^{2+} and 100 mM NH_4^+ in the gradient, there appears the fastest moving component already noted (Fig. 3C, compare Fig. 1A, slice 49). The mobility of this component, which is found predominantly on the slow side of the 30S peak, is only slightly affected by incubation with S1. In experiments analogous to those described in the preceding section (Fig. 2 and Table 1) but with high-salt-washed ribosomes, the fastest 30S species is inactive in MS2 RNA-dependent binding of fMet-tRNA but retains some activity in the other assays of Table 1.

Replacement of E. coli by Caulobacter crescentus S1. As previously reported, *C. crescentus* 30S ribosomal subunits do not form an initiation complex with MS2 RNA (11, 12). In view of the role of S1 reported here it was of interest to ascertain whether this protein is involved in messenger selection. As seen in Table 2, S1 isolated from *C. crescentus* increases, as well as *E. coli* S1, the proportion of the S1-containing *E. coli* 30S subspecies with an accompanying increase of the 30S initiation complex. *C. crescentus* S1 can thus replace the corresponding *E. coli* protein with no change in messenger specificity.

DISCUSSION

This paper shows that only 30S subunits containing protein S1 are able to bind fMet-tRNA$_f$ with MS2 RNA as messenger.

TABLE 2. *Reconstitution of E. coli 30S subunits with protein S1 from E. coli or C. crescentus: Effect on the formation of the initiation complex*

30S ribosomes (A_{260}/sample)	Content of [30S + S1] (%)	MS2-directed binding of f[^{35}S]Met-tRNA	
		Net cpm	Ratio
Untreated (0.570 or 49 pmol)	30	29,420	1.0
Reconstituted with S1 from E. coli (0.580 or 50 pmol)	75	53,200	1.8
Reconstituted with S1 from C. crescentus (0.550 or 48 pmol)	75	45,270	1.6

20 A_{260} units of *E. coli* 30S were incubated (5 min, 37°) with 85 μg of *E. coli* or with 116 μg of *C. crescentus* S1. The mixtures were layered on 5 ml of a 5–20% sucrose gradient containing 20 mM Tris·HCl, pH 7.8/10 mM Mg²⁺/50 mM NH₄Cl/0.1 mM dithiothreitol, and centrifuged for 3 hr at 36,000 rpm and 4° in the SW 65 Spinco rotor. A control without S1 was processed identically. The 30S peak was pooled, assayed as in Table 1, and electrophoresed as in Fig. 2 to determine the [30S + S1] content. Blanks without MS2 RNA were 3200–4200 cpm. The f[^{35}S]Met-tRNA used in this experiment had a specific radioactivity of about 3400 cpm/pmol.

This is consistent with the observation that S1 is indispensable for translation of MS2 RNA by an S1-depleted, cell-free system from *E. coli* (21). Previously (4, 10) it had been shown that S1 is required for MS2 RNA binding to the 30S subunit. Thus, the requirement of S1 for mRNA translation is a consequence of its effect on the ribosomal binding of mRNA. This effect of S1 is not messenger-specific for, as reported earlier (11), *C. crescentus* ribosomes can neither bind nor translate MS2 RNA whereas, as shown here, protein S1 from *C. crescentus* is as active as *E. coli* S1 in initiation complex formation with *E. coli* ribosomes. Specificity in messenger selection by the 30S subunit (11, 22) apparently resides in protein S12 and 16S RNA (23). It is now apparent that base pairing between purine clusters at ribosome binding sites of phage mRNAs and a pyrimidine-rich sequence at the 3′-terminus of 16S RNA (24) may be essential in recognition of messenger initiation regions by the ribosomes. Interestingly, the pyrimidine-rich sequence of 16S RNA of *E. coli* and *Pseudomonas aeruginosa* can form as many as four base pairs with the purine cluster of the coat protein cistron of coliphage (R17) RNA, but the corresponding region of 16S RNA of *C. crescentus* and *Bacillus stearothermophilus* can only form one or two base pairs (25). This would explain the observations on the species specificity of translation of phage mRNA by ribosomes from these bacterial species, i.e., *E. coli* similar to *Pseudomonas; C. crescentus* similar to *B. stearothermophilus* (11, 22, 26). The 30S subunit would appear to interact with mRNA at multiple sites, some of which are specific, e.g., protein S12, 3′-terminus of 16S RNA; some are nonspecific but stabilize the interaction, e.g., protein S1; and some may both stabilize (12) and introduce fine controls in messenger selection, e.g., initiation factor IF3 (18).

As compared to phage mRNA there is less dependence on S1 for both poly(U)-directed ribosomal binding of Phe-tRNA (present work) and poly(U) translation (21). We also found no S1 requirement for AUG-directed binding of fMet-tRNA₅ under our conditions. This may be due in part to the fact

that ribosomal binding of these messengers is facilitated by the much higher concentrations at which they are used relative to phage mRNA.

S1 was considered to be a fractional protein (27), i.e., a protein present in less than one copy per ribosome. However, in agreement with others (3, 19) we find that, with appropriate isolation conditions, the entire 30S population migrates on gels as the [30S + S1] species. S1 is lost either by high salt treatment of ribosomes (0.5–1.0 M NH₄Cl in the washing buffer) or by low salt treatment (below 1.0 mM Mg²⁺ and below 1.0 mM NH₄⁺). Extensive washing with high salt buffers leads to the appearance of a third 30S subspecies which lacks S1 and possibly other proteins (fastest species of Fig. 1A, slice 49). Thus, 30S preparations are mostly mixtures of two or three species, each having somewhat different properties, whose proportions vary with the conditions used for isolation and washing of the ribosomes. S1 is lost from the ribosomes by the same treatments used to extract initiation factors. We have so far not been able to prepare 30S subunits having a full complement of S1 and no initiation factors.

It may be noted that whereas addition of S1 (70,000 daltons) to the [30S − S1] species would increase its molecular weight by approximately 10%, the resulting [30S + S1] species sediments more slowly than [30S − S1]. This suggests that S1 alters the conformation of the 30S subunit.

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