An Essential Function of Ribosomal Protein S1 in Messenger Ribonucleic Acid Translation†

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ABSTRACT: A gentle and efficient method for selectively removing S1 from ribosomes was developed: the S1-free translation system prepared from such ribosomes is stimulated 10–20-fold (depending on the mRNA) by a stoichiometric amount of added purified S1. With this system, we examined the activity of mono- and di-*N*-ethylmaleimide derivatives of S1 in protein synthesis using synthetic and natural mRNAs and electrophoretic analysis of the translation products. The results show that ribosomes containing such modified S1's are functionally active although at a somewhat lower level (50–80% activity). Since treatment of S1 with *N*-ethylmaleimide abolishes the helix-destabilizing ability of S1, we conclude that this ability is not primarily responsible for S1's biological function. A new model for the role of S1 is proposed on the basis of the physical, structural, and RNA-binding properties of S1.

Because of its importance in both protein synthesis and Qβ RNA replication, protein S1[1] is one of the most extensively studied of all ribosomal proteins [e.g., Van Duin & Van Knippenberg (1974), Szer & Leffler (1974), Yokota et al. (1977), Laughrea & Moore (1977), Subramanian et al. (1981), and Schnier et al. (1982)]. It is the largest protein on the *Escherichia coli* ribosome, both in size (557 amino acid residues and M_r 61 × 10^3) and in length (~230 Å) as deduced from a variety of physical and chemical studies (Kimura et al., 1982; Laughrea & Moore, 1977; Giri & Subramanian, 1977; Sillers & Moore, 1981). Protein S1 is apparently made of two distinct structural domains with a freely rotatable region in between (Suryanarayana & Subramanian, 1979; Labischinski & Subramanian, 1979; Chu & Cantor, 1979). The N-terminal third of S1 contains its ribosome binding domain while the central and C-terminal regions, which carry repeating homologous sequences unique among ribosomal proteins, contain the RNA binding domains of S1 [reviewed in Subramanian (1983)].

Protein S1 is a strong RNA binding protein (Lipecky et al., 1977; Draper & Von Hippel, 1978a,b), and many experiments have led to the conclusion that it functions at the mRNA binding step in protein synthesis (Van Dieijen et al., 1976; Thomas & Szer, 1982). Two mechanisms have been proposed to explain the role of S1 in mRNA translation. According to one of them, S1 facilitates the ribosome–mRNA recognition step involving hydrogen bonding between mRNA and its complementary sequence at the 3′ end of 16S rRNA (Dahlberg & Dahlberg, 1975; Steitz, 1979). A functionally important interaction between S1 and the 3′ end of 16S rRNA has however been questioned (Yuan et al., 1979), and actual evidence for the absence of such an interaction in situ has been obtained (Backendorf et al., 1981). The second proposal is based on the observation that S1 is able to unwind double-stranded regions in RNA (Bear et al., 1976; Szer et al., 1976). According to the second view, S1 functions by unwinding double-stranded regions in mRNA and thereby facilitating mRNA–ribosome interaction (Van Dieijen et al., 1976; Thomas & Szer, 1982).

S1 contains two –SH groups, of which one (Cys-349) is more reactive than the other (Cys-292) in the native protein, while both groups are nonreactive in the S1·RNA complex (Subramanian, 1983). Kolb et al. (1977) first observed that the mono-*N*-ethylmaleimide derivative of S1 is unable to unwind nucleic acids [see also Thomas et al. (1978)]. Later experiments with a trypsin fragment and a mutant form of S1 revealed a correlation between the biological activity of the protein and its RNA unwinding ability (Thomas et al., 1979), thus strengthening the second proposal on the mode of action of S1. Other experiments have however weakened this correlation: *N*-ethylmaleimide derivatives of S1 incorporated into ribosomes were found to be functionally active in translating poly(U) (Yokota et al., 1979a) and, incorporated into Qβ replicase, were active in Qβ RNA replication (Cole et al., 1982).

In this paper, we show by amino acid incorporation and product analysis that mono- *and* di-*N*-ethylmaleimide derivatives of S1 are functionally active in translating the coat protein gene of phage MS2-RNA (i.e., a natural mRNA with initiation signals). Thus, the circle of studies is complete that shows that the unwinding property of S1 is not crucial for its biological function. A new model for S1's function on the basis of its physical, structural, and chemical properties [reviewed in Subramanian (1983)] is proposed.

Materials and Methods

Phage MS2-RNA, *E. coli* MRE600 tRNA, poly(U), and poly(A) were purchased from Boehringer [M_r of poly(U) and poly(A) >1 × 10^5]. MS2-RNA was once precipitated with alcohol and dissolved in distilled water before use. ^{14}C-Labeled lysine (335 mCi/mmol), phenylalanine (495 mCi/mmol), and valine (280 mCi/mmol) were from Amersham. *N*-Ethylmaleimide was bought from Sigma.

Ribosomes, supernatant fraction, and initiation factors were isolated from *E. coli* A19 grown in L broth to midlogarithmic phase (Minks et al., 1978). Ribosomal subunits were prepared by zonal centrifugation (Beckman Ti 15 rotor, 25 000 rpm, for 16 h) of *E. coli* A19 S-30 extract (in 10 mM potassium phosphate, 1 mM magnesium acetate and 7 mM 2-mercaptoethanol, pH 7.5) through a 10–38% sucrose gradient. The subunits were collected by pelleting. 30S subunits pre-

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[1] Abbreviations: S1, ribosomal protein S1 of *Escherichia coli*; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride.

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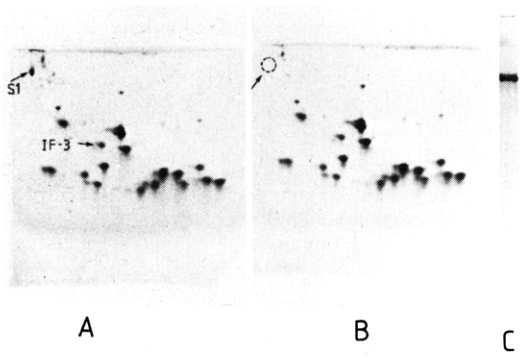

FIGURE 1: Two-dimensional gel analysis of the ribosomal proteins of normal and S1-lacking 30S subunits: (A) normal 30S subunits and (B) S1-lacking 30S subunits, both prepared as described in the text. In each case, protein from 1 A_{260} unit of 30S subunits was electrophoresed (Subramanian, 1974). The arrow in B points to the expected position of S1. (C) Protein S1 used in the present study, electrophoresed according to Laemmli (1970).

pared in this manner contain their normal complement of IF-3 (Figure 1).

Protein S1 was isolated from *E. coli* MRE600 ribosomes by affinity chromatography on poly(U)–Sepharose (Subramanian et al., 1981). The purity of S1 (Figure 1C) was assessed on a sodium dodecyl sulfate–polyacrylamide gel (Laemmli, 1970).

[^{3}H]Uridine-Labeled MS2-RNA. MS2 phage was grown in an 800-mL culture of *E. coli Hfr*C in the presence of 0.5 mCi of [5,6-^{3}H]uridine (New England Nuclear) as described by Billeter & Weissmann (1966). The phage was purified and the RNA extracted as previously described (Minks et al., 1978). The procedure yielded 1.7×10^7 cpm phage RNA with a specific activity of 6400 cpm/μg.

Preparation of f[^{14}C]Met-tRNA$_f^{Met}$. The procedure used for the preparation of f[^{14}C]Met-tRNA was as described by Hershey & Thach (1967), using purified tRNA$_f^{Met}$ (gift from Oak Ridge National Laboratory). The Sephadex G-25 step was replaced by repeated alcohol precipitation. The final f[^{14}C]Met-tRNA had a specific activity of 470 cpm/pmol.

Removal of S1 from 30S Subunits, Supernatant Fraction, and Initiation Factors. Protein S1 present in 30S subunits was removed by affinity chromatography on poly(U)–Sepharose. About 250 A_{260} units of 30S subunits in 10 mM Tris-HCl, pH 7.6, 1 M NH_4Cl, 10 mM magnesium acetate, and 7 mM 2-mercaptoethanol were passed through a 5-mL column of poly(U)–Sepharose (Pharmacia) equilibrated with the same buffer. Washing the column with the equilibrating buffer resulted in elution of 30S subunits free of S1. Protein S1 bound to the column was then recovered by eluting with 7 M urea in buffer. The 30 subunits eluted from the column were completely free of S1 (Figure 1). No other ribosomal protein was missing from these subunits. The removal of S1 from postribosomal supernatant and initiation factor preparations was similarly accomplished by passing them (200 mg of protein) through a 5-mL column of poly(U)–Sepharose. The S1 removal was checked by immuno double diffusion against anti-S1 serum as described previously (Stöffler & Wittmann, 1971). The fractions contained no S1 according to this assay; there were detectable amounts of S1 before passage through the column.

Assay for Protein Synthesis. Translation assays using poly(U) and MS2-RNA have been described previously (Minks et al., 1978; Suryanarayana & Subramanian, 1979). Conditions for poly(A) translation were the same as for poly(U) translation, and hot trichloroacetic acid–sodium tungstate insoluble [^{14}C]lysine was determined. Amounts of ribosome, supernatant protein, initiation factors, and mRNA used are given in the figure legends.

Assays for Binding of MS2-RNA and fMet-tRNA to Ribosomes. Binding of [^{3}H]MS2-RNA to 30S subunits was carried out in a 100-μL reaction mixture containing 50 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl, 10 mM magnesium acetate, 1 mM DTT, indicated amounts of 30S subunits, and 42 000 cpm [^{3}H]MS2-RNA. The mixture was incubated at 37 °C for 10 min, chilled to 0 °C, and then diluted 10-fold with cold binding buffer and filtered through Millipore filters. The filters were washed twice with cold buffer, and the radioactivity retained on the filters was determined in a Beckman LS8000 scintillation counter with toluene–0.5% 2,5-diphenyloxazole as scintillation fluid.

fMet-tRNA binding to ribosomes directed by phage MS2-RNA was carried out in a 100-μL reaction mixture containing 50 mM Tris-HCl, pH 7.1, 60 mM NH_4Cl, 6 mM magnesium acetate, 1 mM DTT, 0.5 mM GTP, 84 pmol of f[^{14}C]Met-tRNA (40 000 cpm), 0.5 A_{260} unit of MS2-RNA, 40 μg of S1-free initiation factors, and 0.8 and 1.8 A_{260} units of S1-free 30S and 50S subunits, respectively. When the effect of S1 was studied, the mixture without GTP and fMet-tRNA was preincubated with S1 at 37 °C, 5 min. The complete reaction mixture was incubated at 37 °C, 10 min, and then filtered through Millipore filters and counted as described above.

Electrophoretic Analysis of in Vitro Translation Products. The reaction mixture from MS2-RNA-directed protein synthesis was incubated with 20 mM Na_2EDTA and 200 μg/mL RNase A at 37 °C, 20 min, and the protein was precipitated with 5 volumes of cold acetone. The precipitate was collected and dissolved in sodium dodecyl sulfate containing sample buffer and electrophoresed in 15% acrylamide slab gels (Laemmli, 1970). The gels were dried and fluorographed (Bonner & Laskey, 1974).

Treatment of S1 with N-Ethylmaleimide. Three different procedures for the reaction of the SH groups of S1 with *N*-ethylmaleimide were carried out. All gave similar results. (i) S1 (1 mg/mL) was incubated with 10 mM DTT at room temperature for 30 min and then dialyzed against 10 mM Tris-HCl, pH 7.4–0.1 M NH_4Cl to remove DTT. It was then treated with a 20-fold molar excess of *N*-ethylmaleimide at room temperature for 30 min and dialyzed against 10 mM Tris-HCl, pH 7.6, 0.1 M NH_4Cl, and 1 mM DTT. (ii) The protein was reacted in the presence of 1 mM DTT by adding a 20-fold molar excess of *N*-ethylmaleimide over total thiols. (iii) In order to react both SH groups of S1 completely, the reaction was done at room temperature in 0.1 M Tris-HCl, pH 7.4, 5 mM Na_2EDTA, and either 8 M urea or 6 M guanidine hydrochloride. After reaction, the protein solution was dialyzed extensively against Tris buffer containing 1 mM DTT.

Reaction of S1 and N-Ethylmaleimide-Treated S1 with 5,5′-Dithiobis(2-nitrobenzoic acid). In order to determine the extent of *N*-ethylmaleimide reaction, S1 or its *N*-ethylmaleimide derivative was precipitated with acetone and the precipitate dissolved in 10 mM Tris-HCl, pH 7.4, 8 M urea, and 1 mM EDTA. The protein (900 μg/mL) was then treated with 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) at room temperature and the absorbance at 410 nm was determined against the appropriate blank.

Miscellaneous. Antibodies to S1 were raised in rabbits and Ouchterlony immuno double diffusion was carried out as described by Stöffler & Wittmann (1971). Protein estimation was according to Lowry et al. (1951), using bovine serum

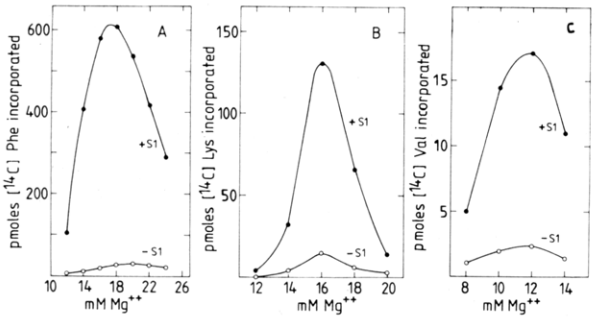

FIGURE 2: Protein-synthesizing activities of the S1-lacking and S1-containing systems described in this paper, as functions of Mg^{2+} concentration: (A) translation of poly(U); (B) translation of poly(A); (C) translation of phage MS2-RNA. For each assay, 44 pmol of S1-lacking 30S subunits, 50 pmol of 50S subunits, 160 μg of S1-free supernatant protein, and 40 μg of S1-free initiation factor fraction (in the case of MS2-RNA) were used. Incubation was for 20 min at 37 °C. The amount of S1, where used, was 4 μg (65 pmol). 1 pmol of [^{14}C]Phe, [^{14}C]Lys, and [^{14}C]Val corresponds to 55, 53, and 232 cpm, respectively.

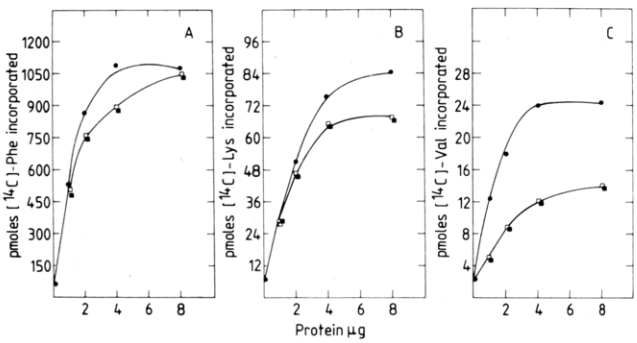

FIGURE 3: Functional activities of S1 and *N*-ethylmaleimide derivatives of S1 in the in vitro translation of (A) poly(U), (B) poly(A), and (C) MS2-RNA. The amounts of ribosomal subunits, supernatant protein, and initiation factors are as given in legend to Figure 2. (●) Untreated S1; (□) *N*-ethylmaleimide- (buffer) treated S1; (■) *N*-ethylmaleimide- (8 M urea) treated S1.

albumin as standard. 1 A_{260} unit of ribosomes or subunits is taken as equivalent to 60 μg.

Results

Translation of mRNA by S1-Free Ribosomes. Figure 2 shows the translation of poly(U), poly(A), and phage MS2-RNA by S1-lacking ribosomes in our S1-free system, with and without added S1, as a function of Mg^{2+} concentration. The translations exhibit sharp Mg^{2+} optima as is generally observed (Van Dieijen et al., 1975; Yokota et al., 1979b). There is a very low level of activity in the absence of S1, which in all cases has the same Mg^{2+} optimum as the S1-containing system. Removal of S1 by the poly(U)–Sepharose column thus gives a clean system with very low background activity and which is stimulated 10–20-fold by the added S1.

Translation of mRNA by Ribosomes Containing N-Ethylmaleimide-Treated S1. Figure 3 shows the activity of *N*-ethylmaleimide-treated S1 in the translation of poly(U), poly(A), and phage MS2-RNA. The treated S1 retains 80–100% of the activity in translating synthetic mRNAs and about 50% of the activity in translating a natural mRNA. The activity of treated S1 was the same when the *N*-ethylmaleimide treatment was done in the presence of 8 M urea or 6 M Gdn-HCl, conditions under which both SH groups of S1 (Cys-292 and Cys-349; Kimura et al., 1982) would be derivatized. Thus, although free SH groups of S1 have a quantitative effect on S1's capacity to translate MS2-RNA, they are not *essential* for translation.

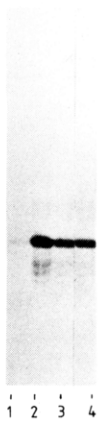

FIGURE 4: Fluorogram of electrophoretically separated translation products of MS2-RNA: (lane 1) protein synthesized by S1-free system; (lanes 2–4) protein synthesized by S1-containing systems; (lane 2) untreated S1; (lane 3) *N*-ethylmaleimide- (buffer) treated S1; (lane 4) *N*-ethylmaleimide- (8 M urea) treated S1.

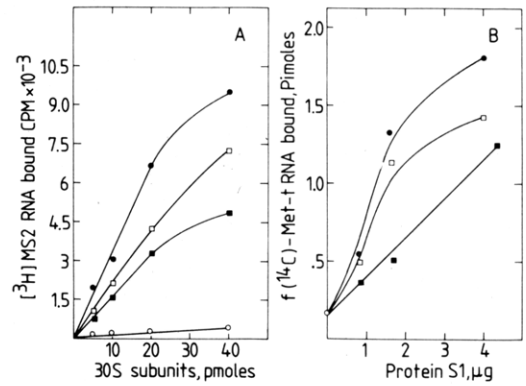

FIGURE 5: Binding of (A) MS2-RNA and (B) fMet-tRNA by ribosomes containing no S1, untreated S1, or *N*-ethylmaleimide-treated S1: (○) binding in the absence of S1; (●) binding in the presence of 4 μg of S1; (□) binding in the presence of 4 μg of *N*-ethylmaleimide- (buffer) treated S1; (■) binding in the presence of 4 μg of *N*-ethylmaleimide- (8 M urea) treated S1.

The results in Figure 4 show that the pattern of translation products obtained with the *N*-ethylmaleimide derivative of S1 is the same as that with S1. Thus blocking of SH groups results in a reduction in the extent of the translation reaction without any *qualitative change* in the nature of translation products.

Binding of MS2-RNA and fMet-tRNA by Ribosomes Containing N-Ethylmaleimide-Treated S1. Figure 5 shows these two properties of the *N*-ethylmaleimide derivatives of S1. It is evident that these results are in accord with the activity of *N*-ethylmaleimide derivatives observed in translation assay with MS2-RNA; i.e., while there is a decrease in the actual binding, derivatized proteins supported the binding at about a 70% level. The *N*-ethylmaleimide treatment was assessed by reacting the S1 and treated S1 with 5,5′-dithiobis(2-nitrobenzoic acid). Under the conditions of the reaction, SH groups of untreated S1 are completely titrated by 5,5′-dithiobis(2-nitrobenzoic acid) while there was either a small (20%) or no reaction, respectively, with S1 treated with *N*-ethylmaleimide in the absence and presence of urea.

Activity of N-Ethylmaleimide-Modified m1S1. m1S1 is a shorter mutant form of S1 that is functionally active in mRNA translation and Qβ replication reactions (Subramanian & Mizushima, 1979; C. Guerrier-Takada, A. R. Subramanian, and P. Cole, unpublished results). While one SH group is more reactive in S1, both SH groups are equally reactive in m1S1 (Subramanian et al., 1981). Experiments with *N*-

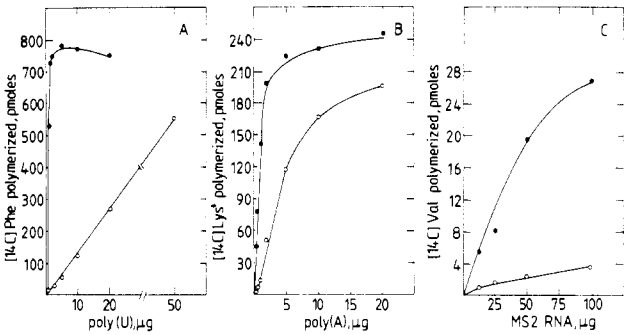

FIGURE 6: Effect of raising mRNA concentration on extent of translation by a fixed amount of ribosomes: translation of (A) poly(U), (B) poly(A), and (C) MS2-RNA. Assays performed as described in the text with the amounts of components as given in the legend to Figure 2. (●) Translation by S1-containing ribosomes and (○) translation by S1-free ribosomes. The counts incorporated in the absence of mRNA [1561, 432, and 301 cpm in the assay systems for poly(U), poly(A), and MS2-RNA, respectively] have been subtracted.

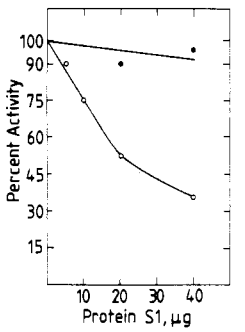

FIGURE 7: Inhibition of poly(A) translation by added excess S1. Translation assay performed with normal 30S subunits; other components as described in the legend to Figure 2. Translation (○) with 2.5 μg/mL poly(A) and (●) with 50 μg/mL poly(A).

ethylmaleimide-modified m1S1 supported the conclusion derived from modified S1. Thus, *N*-ethylmaleimide-treated m1S1 was 80–90% as active as m1S1 in poly(U) and poly(A) translation and about 50% as active as S1 in MS2-RNA translation (data not shown).

Translation by S1-Lacking Ribosomes under Excess mRNA Conditions. In previous experiments, 2 μg of poly(U) and poly(A) and 20 μg of MS2-RNA corresponding to a nominal mRNA/ribosome ratio of 0.5 were used, and under these conditions the S1-lacking system has a very low level of activity (Figure 2). Figure 6 shows that the activity of the S1-lacking system, especially with synthetic mRNA, can be substantially increased by increasing the mRNA/ribosome ratio. This finding supports our proposal (see Discussion) for the role of S1 in mRNA translation.

Inhibition of Poly(A) Translation by Excess S1. Excess free S1 is known to inhibit poly(U) and MS2-RNA translation by binding and sequestering mRNA. This inhibition is relieved by excess mRNA (Van Duin & Van Knippenberg, 1974), but such an effect was not observed with poly(A) translation (Miller & Wahba, 1974; Van Dieijen et al., 1975). We have found (Figure 7) that an inhibition of poly(A) translation can actually be observed provided the concentration of poly(A) used is very low, e.g., 0.25 μg/assay.

Discussion

Two conclusions of general interest on the functions of S1 in protein biosynthesis emerge from the present study. First, it is evident that the modification of one or both of the –SH groups of S1 does not abolish the functional activity of this protein (Figures 3 and 4). Since the blocking of even a single –SH group abolishes the RNA unwinding ability of S1 (Kolb et al., 1977; Thomas et al., 1978), we are led to the conclusion that the unwinding property of S1, although interesting in itself, does not play a primary role in the biological function of S1.

The SH-blocked derivatives of S1 are 80–90% as active as unmodified S1 in the translation of synthetic mRNAs but substantially less active (i.e., ~50%) in the translation of a natural phage mRNA (Figure 3). The *N*-ethylmaleimide derivative of S1 is 80% active in Qβ RNA replication (Cole et al., 1982). Thus while the presence of a bulky group on the sulfur atom of Cys-349 produces only a minor hindrance in the performance of S1's functions in RNA transcription or synthetic mRNA translation, it poses a major hindrance in the translation of phage mRNA. Whether this arises from the complex secondary structure of phage RNA (Steitz, 1975; Fiers, 1975) or because of the additional initiation step in translating a natural mRNA remains to be determined.

The second conclusion is a consequence of the new S1-free system described in this paper. In most of the previous S1-free systems described in the literature (Tal et al., 1972; Van Dieijen et al., 1975; Khanh et al., 1979), the background activity in the absence of added S1 is quite high. For this reason, the observed stimulation by S1 has not been very striking. Our data (Figure 2) show that the stimulation of the S1-free system by added S1, i.e., 10–20-fold, is remarkably high. This biochemical result points to the importance of S1 for the organism. Genetic studies have previously revealed that, unlike many other ribosomal proteins (Dabbs, 1979), S1 may be an essential protein in *E. coli* (Kitakawa & Isono, 1982).

The data summarized in Figure 2 show that our S1-free protein-synthesizing system has a detectable residual activity. We tested for the presence of traces of S1 in this system by gel electrophoresis (Figure 1) and sensitive silver staining (data not shown), as well as by S1 antibodies, but did not detect any S1. Thus, the residual activity seen in Figure 2 is apparently an inherent activity of the S1-lacking ribosome. Previously, we have shown that ribosomes lacking protein L1 are capable of translating mRNA at a reduced, but appreciable level (Subramanian & Dabbs, 1980). The low activity of S1-lacking ribosomes is enhanced greatly when they are provided with a high concentration of mRNA (Figure 6). This phenomenon is particularly striking with respect to synthetic mRNAs. For example, under our assay conditions, S1-containing ribosomes incorporated 550 pmol of phenylalanine when challenged with 0.5 μg of poly(U); the same extent of incorporation was reached by S1-lacking ribosomes with 50 μg of poly(U), i.e., a 100-fold increase in mRNA concentration. Thus, a key consequence of the presence of S1 on the ribosome is a marked reduction in the optimal mRNA concentration for efficient translation.

This finding, when coupled with the known physical, chemical, and structural properties of S1 [reviewed in Subramanian (1983)], leads us to a plausible model for the mode of action of S1. Physical studies have revealed a very elongated, bisegmented shape for S1: it is approximately 230 Å long (same as the maximum dimension of the ribosome) with an apparently freely rotatable hinge between the two segments. Structural studies show a unique sequence at the N-terminal region but a 4-fold repetition of a 74 amino acid residue long sequence in the remaining central and C-terminal regions. The N-terminal region contains the ribosome binding site of S1, while its RNA binding site is located within the structure of the four repeating sequences. In between the two domains are two adjacent, facile trypsin-sensitive peptide bonds that form

a part of the hinge region [see Subramanian (1983) for original references].

These properties, as well as the essential lack of nucleotide specificity in the binding between RNA and S1 (Lipecky et al., 1977; Draper & Von Hippel, 1978a,b), suggest that S1, anchored onto the ribosome at one end of its structure, can search an area around the ribosome for the presence of mRNA. S1 will be able to bind any mRNA that enters its perimeter of action, and the hinged structure would facilitate the subsequent interaction between the ribosome and mRNA, without S1 being directly involved in the latter. This model explains most of the known observations on S1 and its functions as a part of the ribosome, but further experiments on the testable consequences of the model are necessary to establish its validity.

Acknowledgments

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Registry No. Poly(U), 27416-86-0; poly(A), 24937-83-5.

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