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Isolation of 30S and 50S Active Ribosomal Subunits of *Bacillus subtilis*, Marburg Strain

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Active 30S and 50S ribosomal subunits were isolated from *Bacillus subtilis*. These subunits were able to perform not only protein synthesis in the presence of artificial or natural messenger ribonucleic acid but also the specific functions characteristic of each of the subunits. Thus the 30S subunits alone are able to bind formyl-methionyl-transfer ribonucleic acid, and the 50S subunits carry the peptidyl transferase activity.

Studies on the in vitro protein-synthesizing system from *Bacillus subtilis* were heretofore mainly carried out with undissociated ribosomes (2, 4, 6-8, 13, 14, 17, 19).

Few attempts, however, have been made to demonstrate protein-synthesizing ability of dissociated ribosomal subunits of *B. subtilis*. Takeda and Lipmann (17) have shown that the dissociated 30S and 50S ribosomal subunits of this organism are unable to carry out poly(U)-directed polyphenylalanine synthesis. However, by hybridization with complementary ribosomal subunits from *Escherichia coli*, they found that the 30S subunit of *B. subtilis* was preferentially inactivated, whereas the 50S subunit retained considerable activity. Indeed, when in this system the 30S subunit from *B. subtilis* was replaced by the homologous subunit from *E. coli*, synthesis of polyphenylalanine was observed. More recently, Sala et al. (14), utilizing similar methods, found that both "native" and "derived" (dissociated in low Mg^{2+}) ribosomal subunits of *B. subtilis* are unable to promote poly(U)-dependent polyphenylalanine synthesis. Only Wilhelm and Corcoran (19) were able to synthesize polyphenylalanine with recombined 30S and 50S ribosomal subunits from erythromycin-sensitive and -resistant strains of *B. subtilis*. Here too, however, the subunits were not tested for their individual activities.

In the present report we describe the isolation of active 30S and 50S ribosomal subunits which not only perform functions for which intact 70S ribosomes are necessary (protein synthesis) but also the specific functions characteristic of each ribosomal subunit, i.e., formyl-[^{3}H]methionyl-tRNA binding by 30S and peptidyl transferase activity by 50S.

MATERIALS AND METHODS

Growth of bacteria. *B. subtilis* 168 wt, Marburg strain, was grown in tryptone broth in the presence of required salts (6) and harvested at mid-log phase. Cells were washed and stored at −70 C as described previously (12).

Materials. Poly(U) and poly(AUG) were purchased from Boehringer Mannheim. Puromycin was purchased from Nutritional Biochemicals Corp. Folinic acid, Ca^{2+} salt, was from General Biochemicals. [^{14}C]phenylalanine (specific activity, 250 mCi/mmol), [*methyl*-^{3}H]methionine (specific activity, 7 Ci/mmol), [^{14}C]leucine (specific activity, 236 mCi/mmol) and γ-[^{32}P]guanosine 5'-triphosphate (GTP) (specific activity, 2 Ci/mmol) were purchased from C.E.A., Saclay, France. Sucrose (analytical grade) for density gradients was from Prolabo.

Preparation of ribosomes. Ribosomes were prepared and washed three times in high salt as described before (6). For preparation of subunits, the ribosomes were dialyzed against buffer A [tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4), 10 mM; NH_4Cl, 50 mM; and magnesium acetate, 0.1 mM] for 8 to 9 h with two changes of buffer. Samples were layered on a continuous 15 to 30% sucrose density gradient (50 ml) in buffer B (Tris [pH 7.4], 10 mM; NH_4Cl, 50 mM; and magnesium acetate, 0.5 mM) made up over a 5-ml sucrose (50%) cushion dissolved in the same buffer. The sucrose-containing buffers were prepared from a stock of 2 M sucrose solution autoclaved at 120 C for 20 min to destroy contaminating ribonuclease.

Centrifugation was carried out in Spinco SW25.2 rotor at 25,000 rpm for 13.5 to 14 h at 2 C. Fractions of 2 ml were collected. A portion (20 μl) of each fraction was diluted in 1 ml of distilled water, and absorbance at 260 nm (A_{260}) was read. Fractions corresponding to 30S and 50S were collected (see Fig. 1a). The pooled fractions, brought to 10 mM Mg^{2+} by the addition of a 1/100 volume of 1 M magnesium acetate, were centrifuged in a Spinco rotor 65 for 16 h at 60,000 rpm. The resulting pellets

were suspended in buffer C (Tris [pH 7.4], 10 mM; NH_4Cl, 60 mM; magnesium acetate, 10 mM; and 2-mercaptoethanol, 7 mM). Each subunit preparation was stored in small fractions at −70 C. To remove any contamination by 30*S* from the 50*S* fractions, the 50*S* material was repurified through a second similar gradient (see Fig. 1b).

Crude initiation factors. Crude initiation factors were prepared by washing ribosomes with buffer C containing 1 M NH_4Cl. After centrifugation (3 h at 50,000 rpm) of the ribosomal suspension, the upper 4/5 of the supernatant was removed and precipitated with ammonium sulfate (75% saturation). After centrifugation for 30 min at 30,000 × *g* the pellet was dissolved in buffer D (Tris [pH 7.4], 10 mM; NH_4Cl, 250 mM; magnesium acetate, 1 mM; and dithiothreitol, 0.1 mM), dialyzed against the same buffer containing 50% glycerol, and stored at −20 C. To remove contaminating RNA, the preparation was passed through a diethylaminoethyl-cellulose column (1 by 10 cm) equilibrated in buffer D. The fractions (1 ml) presenting a 260/280 ratio of about 0.6 were pooled, precipitated, concentrated, and stored as before.

Preparation of formyl-[^{3}H]methionyl-tRNA. The incubation mixture in a total volume of 1 ml contained the following components: Tris-hydrochloride (pH 7.4), 30 mM; NH_4Cl, 10 mM; magnesium acetate, 10 mM; 2-mercaptoethanol, 5 mM; adenosine 5′-triphosphate (neutralized to pH 7.4), 6 mM; tRNA of *B. subtilis* (unfractionated, prepared as by Capecchi [3]), 2 mg; [*methyl*-^{3}H]methionine (specific activity, 7 Ci/mmol), 40 μCi; tetrahydrofolic acid, 0.4 mM; and a synthetase (prepared according to Vold [18] with the omission of the hydroxylapatite step), 1 mg. The incubation (10 min at 37 C) was stopped by the addition of potassium acetate (pH 5.5; final concentration, 0.2 M). The product was extracted twice by water-saturated phenol and precipitated by alcohol. Under conditions 65 to 70% of [^{3}H]methionyl-tRNA was formylated. One picomole of formyl-[^{3}H]methionyl-tRNA contained 5,600 counts/min. Nonformylated methionyl-tRNA was deacylated by $CuSO_4$ treatment as described by Schofield and Zamecnik (15).

Formyl-[^{3}H]methionyl-tRNA binding assay. The incubation mixture (in 150 μl) contained: Tris-hydrochloride (pH 7.8), 50 mM; NH_4Cl, 75 mM; magnesium acetate, 5 mM; dithiothreitol, 1 mM; GTP, 1.6 mM; poly(AUG) (when added), 0.5 A_{260} units; formyl-[^{3}H]methionyl-tRNA, 5,000 to 7,000 counts/min; crude initiation factor, 15 to 20 μg of protein; and ribosomes or ribosomal subunits as indicated. Incubation was at 30 C for 15 min. The reaction was stopped by adding 3 ml of Tris-NH_4-Mg^{2+} buffer to the above incubation mixture, and the mixture was filtered on cellulose nitrate filters (Schleicher and Schull; 0.45-μm pore size). The filters were washed three times in 3 ml of the same buffer, dried, and counted in toluene in an Intertechnique liquid scintillation counter.

Peptidyl transferase activity. Peptidyl transferase activity was measured according to Miskin et al. (9). Methyl esters were hydrolyzed in 0.6 N KOH for 20 min at 37 C. The reaction product, formyl-[^{3}H]methionyl-puromycin, was extracted with 1.5 ml of ethyl acetate, and 1 ml of the upper phase was counted in a scintillation counter with toluene-Triton as scintillation liquid.

Assays for poly(U)-dependent polyphenylalanine synthesis. Assays for poly(U)-dependent polyphenylalanine synthesis were carried out as described previously (6) in the following incubation mixture (in 125 μl): Tris-hydrochloride (pH 7.8), 52 mM; NH_4Cl, 60 mM; magnesium acetate, 11 mM; dithiothreitol, 4 mM; adenosine 5′-triphosphate, 1 mM; GTP, 0.2 mM; phosphoenolpyruvate, 7.2 mM; pyruvate kinase, 2 μg; spermidine, 0.5 mM; tRNA of *B. subtilis*, 50 μg; 19 amino acids except phenylalanine, 0.1 mM; [^{14}C]phenylalanine, 4.8 μmol (1 pmol = 330 counts/min); poly(U), 25 μg; 150,000 × *g* supernatant; protein, 190 μg; and ribosomes or ribosomal subunits as indicated.

Natural messenger-dependent protein synthesis. Messenger-dependent protein synthesis was carried out in a total volume of 125 μl containing: Tris (pH 7.8), 52 mM; NH_4Cl, 60 mM; magnesium acetate, 11 mM; adenosine 5′-triphosphate, 1 mM; GTP, 0.4 mM; phosphoenolpyruvate, 7.2 mM; pyruvate kinase, 2 μg; tRNA (*B. subtilis*), 50 μg; 19 amino acids except leucine, 10 nmol of each; [^{14}C]leucine (specific activity, 236 mCi/mol; 1 pmol = 470 counts/min), 846 pmol (0.2 μCi); 150,000 × *g* supernatant; protein, 385 μg; and ribosomal subunits as indicated. The messenger RNA (1.4 A_{260} units/assay) used in this study was prepared by infecting *B. subtilis* 168 wt log-phase cells with phage φe (16) at a multiplicity of 5. The cells were harvested 15 min after infection, and RNA was extracted with phenol by the usual method (20).

GTPase activity. Ribosome-dependent elongation factor G, which catalyzed GTPase activity, was carried out according to Nishizuka et al. (10). The assay mixture contained 5 nmol of GTP, 3.35 μg of partially purified elongation factor G (6), and ribosomal subunits as indicated.

RESULTS

It was observed by several investigators (14, 17) that the protein-synthesizing capacity of *B. subtilis* ribosomal subunits was inactivated in vitro. One could assume that this inactivation was the result of exposure of 70*S* ribosomes to dissociating buffer containing only 0.1 mM Mg^{2+}. Table 1 shows that this is not the case. Indeed, exposure of ribosomes to such dissociating buffer for 8 h has practically no effect on the polyphenylalanine-incorporating activity of the 70*S* ribosomes. However, longer dialysis (36 h) against buffer, dissociating or not, dramatically reduces the activity (data not shown).

To obtain active ribosomal subunits several conditions are necessary. (i) The starting 70*S* ribosomal material should have an activity of incorporation of at least 850 pmol of phenylalanine per mg (14.5 A_{260} units) of ribosomes in the

poly(U)-dependent system. (ii) The presence of NH_4^+ ions throughout the isolation procedure is necessary. (iii) The exposure of the ribosomes to low magnesium concentration should not exceed the time necessary for separation of the subunits. Mg^{2+} at a concentration of at least 10 mM should be used for storage of the latter. These criteria were apparently satisfied in the experiment reported by Wilhelm and Corcoran (19).

Figure 1 shows a typical sucrose gradient profile of the 30*S* and 50*S* subunits. The 50*S* subunits were always repurified in a second sucrose gradient and show little contamination by 30*S* subunits. No further purification was necessary for the 30*S* ribosomal subunit.

Poly(U)-dependent polyphenylalanine synthesis with different concentrations of dissociated ribosomal subunits is shown in Fig. 2. The activity is entirely dependent on poly(U). An increase in poly(U) concentration from 25 μg to 100 μg per assay doubles the incorporation of phenylalanine, indicating that poly(U) in the reaction mixture is not present in saturating amount. The 50*S* subunits used in this experi-

TABLE 1. *Polyphenylalanine synthesis by B. subtilis 168 wt ribosomes exposed to low magnesium concentration*[a]

Ribosomes	[^{14}C]phenylalanine incorporated (pmol)	
	− Poly(U)	+ Poly(U)
70*S* undissociated	4	232
70*S* dissociated	1.5	222

[a] Dissociated 70*S* ribosomes were subjected to dialysis (with two changes of buffer) against dissociation buffer (0.1 mM Mg^{2+}) for 8 h. Assay conditions were as described in Materials and Methods. Each assay mixture contained 2.0 A_{260} units of ribosomes. Incubation was for 30 min at 30 C.

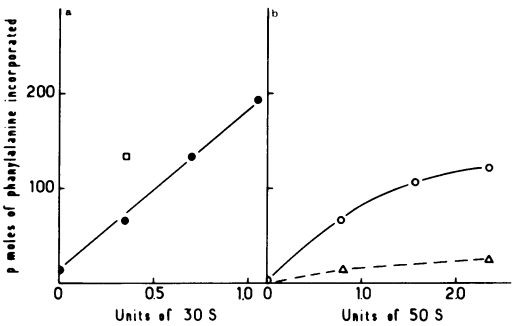

FIG. 2. *Ribosomal subunit-dependent polyphenylalanine synthesis. (a) Reaction contained fixed amount of 50S subunits (0.78 A_{260} units) and increasing concentration of 30S subunits (0.35 to 1.05 A_{260} units); (b) reaction contained fixed amount of 30S subunits (0.35 A_{260} units) and increasing amount of 50S subunits (0.78 to 2.35 A_{260} units). Symbols: ●, 30S subunit-dependent incorporation of phenylalanine; □, same assay but with 100 μg of poly(U) instead of 25 μg (see Materials and Methods); ○, 50S subunit-dependent incorporation of phenylalanine; △, control (phenylalanine incorporation by 50S subunits in the absence of 30S subunit). In the absence of poly (U), 30S and 50S subunits, either separately or together, incorporated less than 1 pmol of [^{14}C]phenylalanine during the incubation period.*

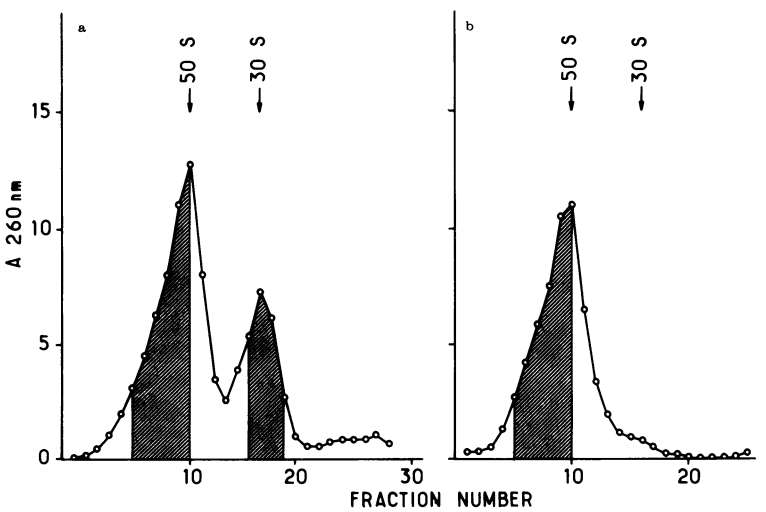

FIG. 1. *Sucrose gradient separation of ribosomal subunits. (a) Profile of dissociated 70S ribosomes. (b) 50S subunits after a second sucrose gradient centrifugation. Hatched regions were pooled, concentrated, and used in the assays. Centrifugation was carried out in an SW25.2 rotor for 13.5 h.*

ment seem to be slightly contaminated by the 30*S* subunits in spite of a second sucrose gradient separation. This probably explains the slight 50*S* concentration-dependent increase of phenylalanine incorporation (Fig. 2b), although the reactions were carried out in the presence of a limiting amount of 30*S* subunit.

Table 2 shows the formyl-[^{3}H]methionyl-tRNA-binding capacity of the ribosomal subunits in the presence and absence of initiation factors. In can be seen that the presence of initiation factors is necessary for binding of formyl-methionyl-tRNA. This is also true for the isolated 30*S* ribosomal subunits and for the 30*S* plus 50*S* subunits taken together.

Table 3 shows the peptidyl transferase activity of the separated subunits as compared to undissociated 70*S* ribosomes. It is evident that the 50*S* component prepared in this study is highly active in peptide bond formation, whereas no activity was detected with the 30*S* subunits.

The dissociated subunits were also able to promote natural messenger-dependent protein synthesis (Table 4). This synthesis was carried out in the presence of 10 to 11 mM Mg^{2+} (optimum for ϕe messenger RNA-dependent protein synthesis). It is known (1) that messenger-dependent protein synthesis at this magnesium concentration can proceed even in the absence of initiation factors. Therefore, we carried out this experiment both in the presence and absence of initiation factors. The results establish that protein synthesis is stimulated almost threefold by initiation factors.

GTPase activity is dependent on the presence of both ribosomal subunits (Table 5). No such activity was found in subunits taken separately.

TABLE 2. *Formyl-[^{3}H]methionyl-tRNA-binding activity by ribosomal subunits*[a]

Ribosomes	Initiation factors	Formyl-[^{3}H]methionyl-tRNA bound (counts/min)	
		-Poly(AUG)	+Poly(AUG)
30*S*	-	73	153
30*S*	+	646	1,208
50*S*	+		230
30*S* + 50*S*	-	82	185
30*S* + 50*S*	+	2,336	2,793
70*S*	+	2,186	2,325

[a] Each assay mixture contained 7,100 counts/min of formyl-[^{3}H]methionyl-tRNA and, when present (+), 20 μg of crude initiation factor. 0.7 A_{260} units of 30*S*, 1.29 A_{260} units of 50*S*, or 0.97 A_{260} units of undissociated ribosomes from the same source as the subunits were used.

TABLE 3. *Peptidyl transferase activity*[a]

Ribosomes	Formyl-[^{3}H]methionyl-puromycin formed (counts/min)
30*S*	24
50*S*	4,167
70*S*	2,645

[a] Ribosomes were added at a concentration of 1.06 A_{260} units of 30*S*, 1.29 A_{260} units of 50*S*, and 1.95 A_{260} units of undissociated 70*S*. The blank (without ribosomes) of 30 counts/min was subtracted from each assay.

TABLE 4. *Natural messenger RNA (mRNA)-dependent protein synthesis by ribosomal subunits*[a]

Ribosomes	Initiation factor	[^{14}C]leucine incorporated (counts/min)		
		-mRNA	+mRNA	Δ mRNA
30*S*	+		338	
50*S*	+		203	
30*S* + 50*S*	+	567	2,112	1,545
30*S* + 50*S*	-	300	837	537

[a] Each assay contained 1 A_{260} unit of 30*S* and/or 1.29 A_{260} units of 50*S*. Twenty micrograms of initiation factor was added when indicated (+). Δ mRNA denotes the difference in the incorporation of [^{14}C]leucine between the presence and absence of messenger RNA.

TABLE 5. *Ribosomal subunit-dependent GTPase activity*

Ribosomes	^{32}P liberated (pmol)
30*S*	0
50*S*	2.5
30*S* + 50*S*	106

[a] Assay contained 3.35 μg of partially purified elongation factor G and 1 and 1.29 A_{260} units of 30*S* and 50*S*, respectively. The amount of ^{32}P liberated (20 pmol) in the presence of elongation factor G alone was subtracted from each case.

DISCUSSION

We have shown here that active ribosomal subunits from *B. subtilis* can be obtained provided that highly active 70*S* ribosomes are used as a starting material. In addition, we have observed, in agreement with the findings of Sala et al. (14), that potassium ions alone are unable to protect ribosomal subunits for their protein synthesizing capacity. The presence of ammonium ions, at a concentration of 30 mM or more, appears to be necessary throughout the isolation procedures. At lower concentrations of NH_4^+

the ribosomal subunits show a gradual inactivation. The activity of these ribosomal subunits can not be restored by heating, as in the case of the inactivated subunits of *E. coli* (9). Long exposure (more than 24 h) to low magnesium concentration also causes inactivation of the subunits.

Isolated active subunits should be able to perform their individual functions as well as the activities which require the presence of the complementary subunit. The *B. subtilis* ribosomal subunits isolated as described here satisfy these criteria. The 30*S* subunits alone are able to bind formyl-methionyl-tRNA, whereas the 50*S* subunits obtained are capable of peptide bond formation. Moreover, the stimulation of formyl-methionyl-tRNA binding by the addition of 50*S* subunits indicates clearly that these two subunits can act together to recycle the initiation factors (5). The functions which need the presence of both the subunits such as protein synthesis (natural or artificial messenger dependent) or elongation factor-catalyzed GTPase are also, at least in part, expressed by these subunits. This may indicate that recombined subunits can be considered as vacant couples of the "tight" variety as defined by Noll et al. (11), i.e., being competent in initiation with natural messenger RNA.

By obtaining active subunits, one major difficulty for characterization of functional ribosomal proteins of *B. subtilis*, which is our ultimate objective, is thus removed.

The preparation of similar active subunits from sporulating cells of *B. subtilis* is in progress. Should this succeed, a detailed investigation on the control of sporulation at the translational level and in particular the possible role of each subunit in this process will be possible.

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