Reconstitution of Bacillus stearothermophilus 50 S Ribosomal Subunits from Purified Molecular Components*

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Bacillus stearothermophilus 50 S ribosomal subunits have been reconstituted from a mixture of purified RNA and protein components. The protein fraction of 50 S subunits was separated into 27 components by a combination of various methods including ion exchange and gel filtration chromatography. The individual proteins showed single bands in a variety of polyacrylamide gel electrophoresis systems, and nearly all showed single spots on two-dimensional polyacrylamide gels. The molecular weights of the proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An equimolar mixture of the purified proteins was combined with 23 S RNA and 5 S RNA to reconstitute active 50 S subunits by the procedure of Nomura and Erdmann (Nomura, M., and Erdmann, V. A. (1970) Nature 226, 1214-1219). Reconstituted 50 S subunits containing purified proteins were slightly more active than subunits reconstituted with an unfraccionated total protein extract in poly(U)-dependent polypeptide synthesis and showed comparable activity in various assays for ribosomal function. The reconstitution proceeded more rapidly with the mixture of purified proteins than with the total protein extract. Reconstituted 50 S subunits containing purified proteins co-sedimented with native 50 S subunits on sucrose gradients and had a similar protein composition. Initial experiments on the roles of the individual proteins in ribosomal structure and function were performed. B. stearothermophilus protein 13 was extracted from 50 S subunits under the same conditions as Escherichia coli L7/L12, and the extraction had a similar effect on ribosomal function. When single proteins were omitted from reconstitution mixtures, in most cases the reconstituted 50 S subunits showed decreased activity in polypeptide synthesis.

METHODS

Buffers—Buffer I: 10 mM Tris-Cl/10 mM MgCl₂/30 mM NH₄Cl/6 mM 2-mercaptoethanol, pH 7.4. Buffer II: same as Buffer I, except 0.3 mM MgCl₂. Buffer III: 30 mM Tris-Cl/20 mM MgCl₂/6 mM 2-mercaptoethanol, pH 7.4. Buffer IV: Buffer III containing 1 mM KCl. Buffer V: Buffer III containing 0.33 M KCl. Buffer VI: 6 M urea/10 mM methyiammonium phosphate/3 mM 2-mercaptoethanol, pH 0.5. Buffer VII: same as Buffer VI, except pH 7.2.

Preparation of Ribosomes, Ribosomal RNA, and Ribosomal Proteins—Ribosomes were obtained from Bacillus stearothermophilus.
Reconstitution of 50 S Ribosomes from Purified Components

strains NO1085, a spontaneous streptomycin resistant mutant of strain 799. The mutant has been used merely to provide a genetic marker to identify the strain, and the mutation has been shown to reside in a 30 S ribosomal subunit (17). Probably the 50 S subunits are the most convenient of those of strain 799 which has been used in the previous studies (5-18).

The growth of the bacteria, the preparation of the ribosomes, and the coaxial separation of ribosomal subunits have been described previously (17).

The 50 S ribosomes were used as a source of 23 S RNA, since the RNA thus obtained is usually more active in reconstitution than RNA extracted from 50 S subunits (18). The 50 S ribosomes were extracted with 4 M urea and 2 M LiCl, and the precipitated RNA was freed of residual protein, as described previously (16). The pelletized RNA was redissolved in 0.05 M Tris-Cl, pH 7.4. This RNA was free of protein, as evidenced by the absence of stained bands when RNA samples were digested with RNase and analyzed by polyacrylamide gel electrophoresis in the "standard" system (see below). The 23 S RNA was purified from 16 S RNA and 5 S RNA either by sedimentation in 5 to 20% sucrose gradients in a Beckman type SW27 rotor or by gel filtration on a column of Bio-Gel A-5m in the same buffer. Conventional methods for preparing RNA often use buffers containing EDTA. However, we found that exposure of 23 S RNA to EDTA made it less efficient for reconstitution. Therefore, EDTA was omitted from all buffers, and in later experiments 3 x 10-4 M MgOAc2, pH 2.0, to remove residual protein, as described previously (16). The pelleted RNA was redissolved from stock solutions of 7.5 M urea which had been purified by treatment with amberlite and charcoal (19).

Ion exchange chromatography was performed with the use of methods similar to those described earlier in connection with the purification of the Escherichia coli 30 S ribosomal proteins (3). Mannan and Dowle standard capacity phosphocellulose (0.9 meq/g, Schwarz/ Mann) or Whatman DE 23 DEAE-cellulose was washed sequentially with 0.5 M NaOH and 0.5 M HCl, freed of fine particles in water, equilibrated in the starting column buffer, and packed into column. Each column was equilibrated with several column volumes of the starting buffer before application of the sample. After the sample was applied, the column was washed with 2 column volumes of the starting buffer before starting the gradient. Flow rates of approximately 17 ml/cm/7 hour were used. The elution profile was monitored by measuring absorbance at 230 nm. Aliquots of the fractions were analyzed for their protein composition by polyacrylamide gel electrophoresis (see below), and appropriate fractions were pooled.

The pooled fractions from the initial large ion exchange columns were concentrated by first diluting them with 2 volumes of Buffer VI, applying this sample to a small phosphocellulose column (1.5 x 7 cm), and eluting the protein with Buffer VI containing 2 M KCl. For subsequent columns, the volumes of the pooled fractions were small enough so that they could be concentrated concomitantly in an Amicon Diaflo ultrafiltration apparatus with UM-2 membranes. If a sample was to be applied to a Sephadex column, it was concentrated by one of the above methods to a protein concentration of greater than 0.5 mg/ml and dialyzed overnight against 3.6 M (NH4)2SO4-76 mM 2-mercaptoethanol. The precipitated protein was collected by low speed centrifugation and dissolved in a small volume of Buffer VI containing 0.3 M KCl to a final volume of 1.5 to 3 ml.

Occasionally dialysis for a few hours against this buffer was required to dissolve the protein. The sample was applied to a column (25 x 100 cm) of Sephadex G-75 or G-100 in Buffer VI containing 0.3 M KCl and eluted with a flow rate of 10 ml/hour.

The proteins were stored frozen, after concentration in the various buffers containing 6 M urea, at -20oC and dialyzed against appropriate buffers just prior to use.

Polyacrylamide Gel Electrophoresis—Three different one-dimensional polyacrylamide gel electrophoresis systems were used for analyzing protein composition. The "standard gel system" refers to the method of Reisfeld et al. (20) as modified by Laboy et al. (21). The separation gels contain 10% acrylamide, 0.15% N,N'-methylenebisacrylamide, and 8 M urea, and electrophoresis is conducted at pH 4.5. The procedure used in this laboratory has been described earlier (22).

A second system is nearly identical with the first dimension of the two-dimensional polyacrylamide gels of Kaltschmidt and Wittmann (23). The separation gel solution contains 4.0 g of acrylamide, 0.15 g of N,N'-methylenebisacrylamide, 3.2 g of boric acid, 0.8 g of 2-mercaptoethanol, 14.1 g of Tris (no urea) per liter, adjusted to a pH of 8.6. Electrophoresis is performed with the cathode at the bottom. The voltage is adjusted to give an initial current of 3 mA per gel, and electrophoresis is continued for 18 hours at constant voltage. The gels are stained in a 1% solution of Amido black in 7.5% acetic acid and 3% aqueous buffers just prior to use.

The third gel electrophoresis system is modeled after the second dimension of the two-dimensional polyacrylamide gels. A separation gel 6 cm high and a stacking gel 1 cm high is used. The separation gel solution contains 10.0 g of acrylamide, 0.44 g of N,N'-methylenebisacrylamide, 6.0 ml of acetic acid, 1.1 ml of 5 M KOH, and 41.1 g of urea in a volume of 100 ml (pH 4.6). Polyacrylamide gel electrophoresis was performed after the cathode at the bottom. The voltage is adjusted to give an initial current of 1.5 mA/gel until the dye bands have passed through the stacking gel and then at a current of 3 mA/gel until the dye bands are 1 cm from the bottom of the gels. The gels are stained in Amido black as described above.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Tran et al. (24). Molecular weights were determined by the method of Shapiro et al. (25), with the use of porcine, carbonic anhydrase, trypsin, soybean trypsin inhibitor, a-lactoglobulin, myoglobin, hemoglobin, ribonuclease A, lysozyme, cytochrome c, and ribonuclease S as standards. The molecular weight of Ribonuclease S was determined in the 10% acrylamide gels by Webers and Osborn (26), with the use of bovine serum albumin, catalase, pyruvate kinase, leucine aminopeptidase, and lactate dehydrogenase as additional standards.

Two-dimensional polyacrylamide gel electrophoresis was performed according to the method of Kaltschmidt and Wittmann (23) or the modified method of Howard and Trotz (27) where indicated. In order to identify the protein number of a purified protein, electrophoretic samples contained about 75 pg of protein, mixed with total protein from 10 A260 units of 50 S subunits to provide a background reference pattern.

Reconstitution—Reconstitution of 50 S subunits was performed according to the method of Nomura and Erdmann (5). Both 23 S RNA and 5 S RNA were dissolved in water. Protein solutions were dialyzed overnight against Buffer IV. For each A260 unit of 23 S RNA, 0.06 A492 unit of 5 S RNA and 2 A140 eq of protein were mixed, and Buffers III and IV were added so that the final concentration was 4 M urea. One A492 equivalent of total protein extract is defined as the amount of protein extracted from 1 A260 unit of 50 S subunits. We define an A492 equivalent of a purified protein as 48 pmol of the protein, since 1 A492 unit of 23 S RNA corresponds to 48 pmol. Protein concentrations were estimated by absorbance at 230 nm. Since 1 A260 unit of total 50 S protein extract contains about 5 pmol of protein (using the Lowry reaction with bovine serum albumin as a standard), we used this average value to calculate the concentrations of each of the purified protein solutions. Thus

1 W. Gette and M. Nomura, unpublished experiments.
A typical reconstitution mixture contained 0.5 $A_{436}$ unit of 23 S RNA and appropriate amounts of other components in a volume of 100 $\mu$l. Unless otherwise indicated, reconstitution mixtures were incubated for 4 hours at 37° and then kept on ice.

**Assays**—In most experiments, the assays for ribosomal activity were performed by taking aliquots directly from reconstitution mixtures in Buffer V, and centrifuging them with the other components of the reaction. For polyphenylalanine synthesis, the volume of the assay mixture was increased and the magnesium concentration adjusted in order to accommodate the presence of a large volume of Buffer V. In the other assays, the amounts of buffer components to be added to the 50 S subunits were calculated in standard proportions that the final concentration of magnesium and univalent salts in the assay mixture, including the contributions from Buffer V, were the same as in the standard procedures.

Poly(U)-dependent polyphenylalanine synthesis was performed by a slightly modified version of a procedure described previously (22). Each incubation mixture contained 75 $\mu$l of native or reconstituted 50 S subunits in Buffer V, 1 $A_{436}$ unit of E. coli 30 S subunits per $A_{436}$ unit of 50 S subunits, 0.5 $\mu$l of pyruvate kinase (6 mg/ml), 2 to 6 $\mu$l of E. coli enzyme fraction (10 to 100 $\mu$g of protein in Buffer I containing 0.25 M KCl) (22), 5 $\mu$l of [U-14C]phenylalanine (20 Ci/mmol, 0.3 M Nh4Cl, 0.3 M 2-mercaptoethanol, 10 $\mu$l of 0.9 M MgCl2, 30 $\mu$l of “Mix,” and enough Buffer I to bring a final volume of 200 $\mu$l. One milliliter of “Mix” contains 10 $\mu$l of 2 M Tris-Cl, pH 7.4, at 20°, 20 $\mu$l of 1 M MgCl2, 47 $\mu$l of 2 M NH4Cl, 114 $\mu$l of 40 mM ATP, 30 $\mu$l of 3 mM GTP, 1 $\mu$l of 2-mercaptoethanol, 70 $\mu$g of dithiothreitol, 100 $\mu$l of 0.1 M phosphonopyruvic acid tricyclohexylammonium salt, 3.3 mg of tRNA from E. coli strain B (Grand Island Biological Co.), enough KOH to bring the pH to 7.4, and H2O to a total volume of 1 ml. The final magnesium concentration in the reaction mixture was 18 $\mu$M, since this concentration was found to be optimal for both native and reconstituted 50 S subunits at this KCl concentration (0.125 M). The mixtures were incubated at 37° for 30 min, and the reaction was stopped by the addition of 2 ml of 5% trichloroacetic acid. The acid precipitates were heated in a boiling water bath for 15 min, brought to room temperature, and filtered onto Reeve Angel glass fiber filters (grade 343AH). The filters were dried and placed in scintillation vials with 5 ml of scintillation fluid (100 g of 2,5-diphenyloxazole (PPO) and 4 g of 1,4-bis[5-(phenyloxazolyl)]benzene (POPOP) per liter of toluene), and the radioactivity was measured in a liquid scintillation counter.

Pepidyltransferase assay mixtures contained 50 S subunits in 50 $\mu$l of Buffer V, 5 $\mu$l of 0.3 M Tris-Cl, pH 7.4, at 20°, 60 mM MgCl2, 1.56 M KCl, 15 $\mu$l of microsomal tRNA (20 Ci/mmol) in 1 ml, 44 ml of H2O, and 50 $\mu$l of 1 mM puromycin in methanol. The assay was performed and the tRNA purified as described previously (17). The aminoacyl-tRNA-binding (tRNA protection) assay mixtures contained 50 S subunits in 50 $\mu$l of Buffer V, 5 $\mu$l of 0.2 M Tris-Cl, pH 7.4, at 20°, 0.26 M MgCl2, 10 $\mu$l of Buffer I containing 1 $A_{436}$ unit of E. coli 30 S subunits per $A_{436}$ unit of 50 S subunits, 30 $\mu$l of [14C]Phe-tRNA, 440 Ci/mol, prepared according to Nishizuka et al. (30), and enough H2O to give a final volume of 100 $\mu$l. The assay was performed as described previously (17).

The EF-G-dependent GTPase assay was based on the procedure of Marsh and Parmeggiani (29). Assay mixtures contained 50 S subunits in 20 $\mu$l of Buffer V, 5 $\mu$l of 0.75 M Tris-Cl, pH 7.4, at 20°, 60 mM MgCl2, 0.2 M NH4Cl, 0.3 M 2-mercaptoethanol, 2 $\mu$l of Buffer I containing 1 $A_{436}$ unit of E. coli 30 S subunits per $A_{436}$ unit of 50 S subunits, 5 $\mu$l of 1 mM GDP, and enough H2O to give a final volume of 100 $\mu$l. The reaction was started by the addition of 20 $\mu$l of 3 M HC1O4. Five microliters of 20 mM KH2PO4, 0.3 ml of 0.02 M sodium molybdate, and 0.4 ml of isopropyl alcohol were added. Each tube was shaken and centrifuged to remove a minor precipitate, and the supernatant was withdrawn and mixed with 5 ml of scintillation fluid, and the radioactivity was measured in a scintillation counter.

Reconstitution of 50 S Ribosomes from Purified Components

**RESULTS**

Two-dimensional Polyacrylamide Gel Electrophoresis and Protein Nomenclature—Fig. 1a shows a two-dimensional polyacrylamide gel electrophoresis pattern of Bacillus stearothermophilus 50 S proteins, with electrophoresis in 4% acrylamide in the first dimension. Fig. 1b shows a similar pattern for a different preparation of 50 S subunits, with electrophoresis in 8% acrylamide in the first dimension and shorter electrophoresis times in both dimensions so that Proteins 37 and 38 remain on the gel. The numbers were assigned simply by numbering the spots on a similar pattern several years ago in this laboratory. The nomenclature appeared in a previous publication (12).4 Two points should be made. First, the electrophoresis pattern is different from that of Escherichia coli 50 S subunits, and no attempt was made to label analogous proteins from the two species with the same number. For example, it has been shown that B. stearothermophilus Protein 3 is immunologically related to E. coli L2 (36). Second, the numbering of spots from 1 to 38 does not imply the existence of 38 proteins. The numbers listed correspond to the proteins that have been purified in the present study. Several numbers in the original numbering system correspond to faint spots which have not been observed by us or to spots which we do not regard as distinct proteins but rather as derivatives of proteins present in other spots. The individual cases will be taken up under “Discussion.”

The gel in Fig. 1b also shows the presence of several small acidic spots near the top of the gel. These spots vary in intensity from one sample to the next but are always faint. Similar spots are seen in E. coli 30 S and 50 S subunits. Presumably they represent large supernatant proteins which adhere to ribosomal subunits. The gel pattern also reveals faint spots to the upper left and lower right of Protein 30. These spots were always present but were always quite faint, and both underwent co-electrophoresis with 30 S proteins, the upper spot appearing in the region of B. stearothermophilus S15 and S17, and the lower spot appearing at the position of B. stearothermophilus S20 (nomenclature of Higo et al., Ref. 34). They are probably small quantities of 30 S proteins which adhere to 50 S subunits (cf. Ref. 37). The spot to the upper right of Protein 25 was

*Previously, we attached the prefix “L” to the protein number, e.g., L1, L2, etc., in analogy to the nomenclature used for Escherichia coli ribosomal proteins (32). A different numbering system, also using the L prefix, has appeared, based on the two-dimensional gel pattern for Bacillus subtilis (35). In this publication we refer to the proteins by numbers alone. We feel that a tentative numbering system should be adopted until the structural and functional correspondence of each of these proteins with one of the E. coli 50 S proteins has been established, and then an L number should be assigned on the basis of this correspondence. For example, the 30 S ribosomal proteins of Bacillus stearothermophilus have been studied by two groups. In our laboratory (34), a name was assigned to a protein, for example, S5, only when the protein was shown to be chemically or functionally (or both) related to E. coli protein S5. Isozo et al. (55) numbered the proteins according to their positions on two-dimensional gels. Isozo's B5S corresponds to E. coli B4 and was called B. stearothermophilus S4 by our group. The adoption of a tentative numbering system before homologies with E. coli proteins are established can help to avoid this type of confusion.
absent in most preparations of 50 S subunits. No other faint spots reproducibly appeared in the gel patterns.

The electrophoresis pattern of 50 S subunits was similar whether the 70 S ribosomes used as the starting material were washed with sucrose and 0.5 M KCl or simply obtained as a crude pellet from an S30 extract. Therefore, no 50 S ribosomal proteins were removed in substantial quantities by the washing procedure. Often the spots due to supernatant proteins referred to in the last paragraph were of lesser intensity in subunits derived from salt-washed ribosomes.

One-dimensional Polyacrylamide Gel Electrophoresis—Fig. 2 shows an electrophoresis pattern of RNase-digested 50 S subunits in the “standard gel system” (see “Methods”). The proteins are resolved into many bands. Some of the bands contain two proteins, and one band, the very dark band in the middle of the pattern, contains six different proteins. The group of bands near the top of the gel contains several different large acidic proteins, among them, Protein 1. (Identification of the proteins in each band was done in the course of the present study by the two-dimensional gel electrophoresis of purified or partially purified protein preparations; see below.) In some cases, the proteins undergoing co-electrophoresis were well separated on primary phosphocellulose columns, and resolution of the proteins on one-dimensional gels was not necessary for the subsequent purification. In other cases, proteins undergoing co-electrophoresis were not resolved in the initial stages of the purification. Since it was too laborious to perform two-dimensional electrophoresis routinely on column fractions, we devised one-dimensional gel systems similar to the first and second dimensions of the two-dimensional gels to analyze column fractions in these cases.

Such gels are shown in Fig. 3. Electrophoresis at pH 8.6 in gels containing 4% acrylamide, corresponding to the first dimension of two-dimensional gels, separated Protein 23 from Protein 28 and Protein 22 from Protein 26. Electrophoresis at pH 4.6 in gels containing 14% acrylamide, corresponding to the second dimension of two-dimensional gels, separated Protein 8 from Protein 13 and Protein 30 from Protein 32. Finally, Proteins 20a and 20b, which underwent co-electrophoresis in all three of the urea gel systems, were resolved in sodium dodecyl sulfate gels.

Protein Purification—A schematic diagram of the initial stages of the purification is shown in Fig. 4. The procedure followed methods which have been described earlier (17). 50 S units (122,000), or about 9 g, of 50 S subunits were purified by zonal centrifugation from salt-washed 70 S ribosomes. The subunits were extracted with 4 M urea and 2 M LiCl, and the precipitated RNA was re-extracted with 4.5 M urea/0.5 M Mg(OAc)₂, pH 2.0 (see “Methods”). The supernatant from the urea/LiCl extraction contained only trace amounts of Protein 3, all of the other proteins, and most of the 5 S RNA. The supernatant from the second extraction contained nearly all of Protein 3 and small amounts of Protein 6.
Reconstitution of 50 S Ribosomes from Purified Components

FIG. 3. Mixtures of the proteins indicated were subjected to electrophoresis as follows: a and b, 4% acrylamide, pH 8.6; c and d, 14% acrylamide, pH 4.6; e, sodium dodecyl sulfate gel.

The supernatant (600 ml) from the first extraction was dialyzed overnight against 3.5 liters of Buffer VI, and a small amount of precipitate formed inside the dialysis bag. This precipitate was removed by centrifugation, and the solution was again dialyzed against Buffer VI containing 0.25 M KCl. The precipitate was dissolved in 7.5 M urea containing 10 mM phosphoric acid and was dialyzed against Buffer VI containing 0.3 M KCl. It was found to contain about 75 mg of protein consisting of roughly equal amounts of Proteins 20b, 25, and 37 or 38 (or both). Most of these proteins remained in solution. This precipitation occurred to varying extents in subsequent experiments. Sometimes, the KCl concentration could be lowered to 0.005 M without any precipitation of proteins, and on other occasions even more extensive precipitation of these three proteins occurred if the KCl concentration was decreased below 0.2 M. We do not know what conditions affect the amount of precipitation.

The protein solution was divided into two equal parts, and each was applied to a phosphocellulose column (2.5 x 100 cm) equilibrated with Buffer VI containing 0.25 M KCl. Each column was washed with 1 liter of Buffer VI containing 0.25 M KCl, eluted with 8 liters of Buffer VI with a linear gradient of 0.25 to 0.6 M KCl, and washed with 1 liter of Buffer VI containing 0.6 M KCl followed by 1 liter of Buffer VI containing 0.8 M KCl. The elution profile as monitored by absorbance at 290 nm and the identities of the proteins in the various peaks are shown in Fig. 5. The columns gave identical elution profiles, and corresponding fractions from the two columns were pooled and concentrated as described under "Methods." The pass-through fractions from the two columns were combined, dialyzed against 3 volumes of Buffer VI, and then against 3 volumes of Buffer VI containing 0.05 M KCl. The solution was applied to a phosphocellulose column (2.5 x 100 cm) equilibrated in Buffer VI containing 0.05 M KCl, and eluted with 4 liters of Buffer VI with a linear gradient of 0.05 to 0.25 M KCl. The elution pattern is shown in Fig. 6.

The pH of the pass-through from this column was raised to 7.2 by the addition of methylamine and applied to a DEAE-cellulose column (2.5 x 100 cm) equilibrated with Buffer VII containing 0.05 M KCl. The column was washed with 600 ml of Buffer VII containing 0.05 M KCl and eluted with 4 liters of Buffer VII with a linear gradient of 0.05 to 0.3 M KCl, then washed with 500 ml of Buffer VII containing 1 M KCl. The elution pattern is shown in Fig. 7. The peak eluting at 1 M KCl was the source of the 5 S RNA used in the reconstitution experiments.

Appropriate fractions from these columns were pooled and concentrated as described under "Methods," and the proteins corresponding to the various bands on one-dimensional gels were identified by two-dimensional polyacrylamide gel electrophoresis. A few of the proteins, labeled nr on the elution patterns, corresponded to the large non-ribosomal supernatant proteins referred to earlier. The elution profiles also revealed small amounts of proteins which did not correspond to any spot on the two-dimensional gel pattern, presumably 30 S proteins or non-ribosomal contaminants. These are labeled (30 S) on the elution profiles.

Figs. 5 to 7 reveal that several of the proteins were obtained partially or completely in pure form from these initial phosphocellulose and DEAE-cellulose columns, but most of the proteins required further purification. This was achieved, whenever possible, by gel filtration on Sephadex columns and in a few cases by chromatography on phosphocellulose at pH 8.5. A summary of the secondary column procedures is shown in Fig. 8.
contaminants present in the original columns were removed. Phosphocellulose elution profile revealed that many of the minor proteins resolved on Sephadex G-100 as shown in Fig. 8. The phosphocellulose elution profile revealed that many of the minor proteins were partial except that Proteins 21 and 10 were better resolved, emerging as a clear double peak. Proteins 8 and 11 were partially inhibited, activity in reconstitution experiments. The purified protein did not stimulate, and often slightly inhibited, activity in reconstitution experiments. The purified protein was established by two-dimensional polyacrylamide gel electrophoresis. All proteins showed single spots on two-dimensional gels, with the exception of Proteins 8, 13, 30, 31, 32, 37, 38. As discussed below, we feel that the multiple spots present in these cases represent derivatives of a single protein.

We encountered considerable difficulties in the separation of Proteins 8, 11, and 13 from the pass-through of the DEAE-cellulose column. Therefore, the following procedures were used to purify these three proteins. The 50 S subunits were first extracted with ethanol in the presence of 0.5 M NH₄Cl by the procedure of Hamel et al. (31). The supernatant contained Protein 13 along with minor amounts of other proteins which were removed by chromatography on DEAE-cellulose by the procedure of Müller et al. (38). The precipitated 50 S subunits were redissolved and the protein extracted with 4 M urea/2 M LiCl. The protein was dialyzed extensively against Buffer VI until the KCl concentration was 0.003 M, and this time no precipitation occurred. The protein was applied to a column of phosphocellulose equilibrated with Buffer VI containing 0.003 M KCl and eluted with a gradient of 0.008 to 0.7 M KCl in Buffer VI. Proteins 8 and 11 emerged in the pass-through, Protein 1 and a non-ribosomal protein emerged in separate peaks at the beginning of the gradient, and the remainder of the elution profile was similar to those shown in Figs. 5 and 6, except that Proteins 21 and 10 were better resolved, emerging as a clear double peak. Proteins 8 and 11 were partially resolved on Sephadex G-100 as shown in Fig. 8a. The phosphocellulose elution profile revealed that many of the minor contaminants present in the original columns were removed along with Protein 13 by the ethanol/NH₄Cl extraction.

Although the columns depicted in Figs. 5 and 6 were the source of nearly all of the purified proteins used in the reconstitution experiments, the procedure just described (that is, prior extraction of Protein 13, subsequent extraction of the other proteins with urea/LiCl, and chromatography on a single phosphocellulose column with a gradient beginning at 0.003 M KCl) appears to be a better, more straightforward procedure and avoids the difficulties encountered in the first procedure in separating Proteins 8, 11, and 13.

Purity and Identity of Proteins—The purity of the individual protein preparations was established by polyacrylamide gel electrophoresis with the use of the three systems described under “Methods” as well as sodium dodecyl sulfate gels and two-dimensional polyacrylamide gels. Gel patterns of the purified proteins using the “standard gel system” are shown in Fig. 9. The gels demonstrate that nearly all of the proteins are at least 95% pure, with the possible exception of Protein 8. This protein contains perhaps 10% Protein 11. Fig. 10 shows electrophoresis patterns for Proteins 8, 13, 22, 23, 26, 28, 30, and 32, with the use of the appropriate gel systems in order to show that these proteins are free of contaminants which would appear in the same band in the standard gel system. In addition, all of the proteins showed single bands on sodium dodecyl sulfate gels except for Protein 8 which contained a minor band of Protein 11 (see above). The identity of each protein was established by two-dimensional polyacrylamide gel electrophoresis. All proteins showed single spots on two-dimensional gels, with the exception of Proteins 8, 13, 30, 32, 37, and 38. As discussed below, we feel that the multiple spots present in these cases represent derivatives of a single protein.

The yields of the purified proteins were quite variable, about 60 mg in the best cases and as low as 10 mg in the worst, compared to a theoretical yield of about 60 mg per 10,000 molecular weight.

Molecular Weights of Proteins—The molecular weights of the proteins were determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate solutions, as described under “Methods.” The results are presented in Table I. The distribution of molecular weights is similar to that of the E. coli 50 S ribosomal proteins (2), except that E. coli has no protein corresponding to Protein 1 with a molecular weight of 5.3 × 10⁶.

Reconstitution of 50 S Subunits Using Purified Proteins—An equimolar mixture of the purified proteins was obtained by mixing together amounts of the individual proteins proportional to their molecular weights, with the following exceptions. The mixture contained 2.5 mol of Protein 13 per mol of each of the other proteins, since E. coli 50 S subunits contain 2 to 3 copies of the analogous protein L7/L12 (39). Also, the mixture contained a 3-fold excess of Protein 20b since preliminary experiments showed that this amount was necessary in order to obtain maximal activity in reconstitution experiments. This could be due to a partial inactivation of the Protein 20b preparation during the purification. Also, Protein 37 was omitted since preliminary experiments showed that the presence of this protein did not stimulate, and often slightly inhibited, activity in reconstitution experiments. The purified protein mixture was dialyzed overnight against Buffer IV.

This mixture of purified proteins was compared with a total protein extract, prepared by extraction of 50 S subunits as described under “Methods,” for its ability to form 50 S subunits when incubated with 23 S RNA and 5 S RNA under reconstitution conditions. Fig. 11 shows titration curves for
Reconstitution of 50 S Ribosomes from Purified Components

FIG. 9. Electrophoresis of purified proteins in the standard gel system. Since the various gels were run at different times, the relative positions of the proteins are not always the same as in Fig. 2. The weak bands near the bottoms of the gels for Proteins 20A, 24, and 32 are due to the tracking dye.

FIG. 10. Gel electrophoresis of purified proteins. Proteins 8, 13, 30, and 32 were subjected to electrophoresis in gels containing 14% acrylamide, pH 4.6, and Proteins 22, 23, 26, and 28 in gels containing 4% acrylamide, pH 8.56. The weak tracking dye bands are seen in the gels for Proteins 8, 13, 30, and 32.

reconstitution experiments using the two protein mixtures. In this experiment, increasing amounts of each protein mixture were added to a constant amount of 23 S RNA and 5 S RNA, reconstitution was performed, and aliquots of the reconstitution mixtures were assayed directly for their activity in poly(U)-dependent polyphenylalanine synthesis. Fig. 11 shows that the mixture of purified proteins was actually slightly more active in reconstitution than the total protein extract, and that a plateau value was reached at a slightly lower molar ratio of proteins to RNA, about 1.5 instead of about 1.8. The fact that the activity plateaus at a molar ratio of proteins to RNA greater than 1.0 could be due to errors in estimating protein concentrations or molecular weights or partial inactivation of some of the proteins. This experiment demonstrates that the mixture of purified proteins does not lack any component present in the total protein extract which is essential for ribosome activity in poly(U)-dependent polyphenylalanine synthesis.

Fig. 12 compares the kinetics of reconstitution using the two protein preparations, each at a concentration of 2.0 mol/mol of RNA, a value in the plateau regions of the titration curves of Fig. 10. The mixture of purified proteins showed faster reconstitution than the total protein extract. This difference was reproducible in several experiments. This experiment indicates that the mixture of purified proteins does not lack any component which acts to accelerate the rate of subunit reconstitution, as was observed for the E. coli 30 S Protein S16 (40). The reason for faster reconstitution with purified proteins has not been investigated.

Sucrose gradient sedimentation was used to compare the sedimentation coefficients of 50 S subunits reconstituted with the purified protein mixture with that of native 50 S subunits. Fig. 13 shows that the bulk (about 60%) of the reconstituted 50 S subunits co-sedimented with native 50 S subunits. In addition, reconstituted 50 S subunits showed a somewhat greater tendency to aggregate than native 50 S subunits. A small amount of slowly sedimenting material, probably due to degradation of the 23 S RNA, was also present.

The protein composition of the reconstituted 50 S subunits

TABLE I

Molecular weights of 50 S ribosomal proteins

Molecular weights were determined by sodium dodecyl sulfate gel electrophoresis as described under “Methods.” Numbers are averages of two determinations.

| Protein | M, x 10^-3 | Protein | M, x 10^-3 |
|---------|------------|---------|------------|
| 1       | 53.0       | 21      | 15.4       |
| 2       | 28.2       | 22      | 14.6       |
| 3       | 33.2       | 23      | 14.4       |
| 4       | 28.7       | 24      | 15.5       |
| 5       | 24.2       | 25      | 20.4       |
| 6       | 28.2       | 26      | 14.3       |
| 8       | 19.2       | 28      | 13.9       |
| 10      | 24.0       | 29      | 14.3       |
| 11      | 18.4       | 30      | 13.7       |
| 13      | 13.0       | 32      | 10.6       |
| 15      | 18.5       | 34      | 12.7       |
| 18      | 14.7       | 37      | 12.8       |
| 20a     | 17.1       | 38      | 12.8       |
| 20b     | 18.2       |         |            |
Reconstitution of 50 S Ribosomes from Purified Components

Fig. 11. Titration of RNA with proteins. Each reconstitution mixture contained 0.4 $A_{260}$ unit of 23 S RNA, 0.025 $A_{260}$ unit of 5 S RNA, and the amount of total protein extract (O—O) or purified protein mixture (■—■) indicated on the abscissa in a volume of 180 µl. Duplicate 75-µl aliquots of each reconstitution mixture were assayed for polyphenylalanine synthesis. An equivalent amount of 50 S subunits polymerized 100.2 pmol of phenylalanine (7492 cpm).

Fig. 12. Kinetics of reconstitution. Reconstitution mixtures contained 0.585 $A_{260}$ unit of 23 S RNA, 0.035 $A_{260}$ unit of 5 S RNA, and 1.17 $A_{260}$ eq of either total protein extract (O—O) or purified protein mixture (■—■) in a volume of 180 µl. The mixtures were incubated at 60° for the times shown, and duplicate 75-µl aliquots of each reconstitution mixture were assayed for polyphenylalanine synthesis. An equivalent amount of 50 S subunits polymerized 141.8 pmol of phenylalanine (10,931 cpm).

was determined by two-dimensional polyacrylamide gel electrophoresis. The results are shown in Fig. 14. The reconstituted particles contained all the proteins added in the reconstitution mixture except that no spot appeared in the position of Protein 38. Instead, a very faint spot appeared to the left of the position for Protein 38, in the position previously labeled Spot 36. This spot probably represents a derivatized form of Protein 38 (see under “Discussion”). In this gel, Proteins 4 and 26, which have isoelectric points close to 8.56, migrated in the acidic direction, and Proteins 24 and 25 were poorly resolved.

Finally, the reconstituted particles were assayed for their activity in various partial activities of protein synthesis, peptidyltransferase, EF-G-dependent GTPase, EF-Tu-dependent GTPase, and ability to protect tRNA from ribonuclease digestion in a 70 S complex. This last assay (41) is a measure of the ability of 50 S subunits to associate with 30 S subunits to form functional 70 S couples. In this experiment, the particles were concentrated by pelleting before assay. We found that pelleting the reconstituted particles did not cause...
Reconstitution of 50 S Ribosomes from Purified Components

Table II

| Activity                      | 50 S Subunits |          | Total protein |          | Purified proteins |          |
|-------------------------------|---------------|----------|---------------|----------|------------------|----------|
|                               | cpm    | pmol | %    | cpm    | pmol | %    | cpm    | pmol | %    |
| Poly(Phe) synthesis          | 15,423 | 100.6 | 100 | 5,107 | 34.0 | 33 | 6,095 | 40.9 | 34 |
| Direct assay                  |          |        |      |          |        |    |        |      |    |
| Pelleted particles            | 4,531 | 0.190 | 100 | 584 | 0.024 | 13 | 456 | 0.10 | 10 |
| Peptidyltransferase           | 1,968 | 2.98 | 100 | 491 | 0.74 | 25 | 437 | 0.66 | 22 |
| EF-G GTPase                   | 2,422 | 25.4 | 100 | 780 | 8.2 | 32 | 962 | 10.1 | 40 |
| EF-T GTPase                   | 2,259 | 0.305 | 100 | 702 | 0.095 | 31 | 774 | 0.105 | 34 |

Any decrease in activity in polyphenylalanine synthesis, and actually caused a slight increase. The other assays were performed only with pelleted particles. The results show that 50 S subunits reconstituted with the mixture of purified proteins had activities comparable to 50 S subunits reconstituted with the total protein extract in all the assays, slightly higher activity in polyphenylalanine synthesis, EF-G-dependent GTPase, and EF-T-dependent GTPase, and slightly lower activity in peptidyltransferase and Phe-tRNA protection. The values for percentage of reconstitution compared to native 50 S subunits, as examined by the various assays, were comparable, except that the reconstitution of peptidyltransferase activity was less efficient than the reconstitution of other ribosomal activities.

Many reconstitution experiments have been done using purified 50 S components. The efficiency of reconstitution determined by the activity in poly(U)-dependent polyphenylalanine synthesis varied from about 20% to as high as 50% with various preparations of 23 S RNA (33% in Table II). Failure to get 100% efficiency is at least partly due to incomplete reconstitution of physically intact 50 S particles (cf. Fig. 13). Breakdown of some 23 S RNA appears to take place during reconstitution to a variable extent.

Ethanol/NH₄Cl Extraction Experiments—Hamel et al. (31) showed that precipitation of E. coli 50 S subunits with ethanol in the presence of 0.5 M NH₄Cl released a protein which was essential for polyphenylalanine synthesis and specifically for all reactions involving elongation factors G and T. This protein was identified as E. coli L7/L12 (42-44). We performed a similar experiment with B. stearothermophilus 50 S subunits to see if they contained a protein with similar properties. 50 S subunits were extracted by the procedure of Hamel et al. (31), and the extracted protein was isolated. Two-dimensional polyacrylamide gel electrophoresis (not shown) revealed that both of the spots labeled 13 (see Fig. 1) were completely removed by the extraction procedure. The relative intensities of these spots vary among different samples of subunits. This particular sample had a large amount of the left hand spot and a smaller amount of the right hand spot. The protein extracted by the ethanol treatment showed only the left hand spot.

Reconstitution experiments with these preparations are presented in Table III. The extraction procedure led to a severe loss of polyphenylalanine-synthesizing activity and a complete loss of EF-G-dependent GTPase activity. Both activities were nearly completely restored by adding back the extracted protein. These experiments demonstrate that B. stearothermophilus Protein 13 is extracted from 50 S subunits under conditions similar to those employed for E. coli L7/L12, and that the extraction has similar effects on ribosomal activity.

Incidentally, we have found that ethanol precipitation of 50 S subunits from concentrated sucrose solutions also results in the removal of Protein 13. Apparently, sucrose and NH₄Cl have similar effects in facilitating the extraction of this protein. About 80% loss of polyphenylalanine-synthesizing activity occurs when 50 S subunits are precipitated from solutions containing 38% (w/w) sucrose (the solution used in zonal centrifugation), while no loss of activity occurs if the sucrose is diluted to a concentration of less than 10% (w/w) before the addition of ethanol.

Single-component Omission Experiments—In order to determine whether the purified proteins are individually required for the activity of reconstituted 50 S subunits, reconstitution was performed with 23 S RNA, 5 S RNA, and mixtures of proteins in which a single protein was omitted in each case. These mixtures contained all of the purified proteins except Protein 37, for the reasons mentioned above. In addition, early experiments indicated that Protein 1 had no effect on the activity of reconstituted particles, except that in a few experiments the addition of this protein led to inhibition of activity as high as 30%. Therefore, Protein 1 was also omitted in the later experiments. The reconstituted particles were assayed for their activity in poly(U)-dependent polyphenylalanine synthesis. The results are summarized in Table IV. They indicate that the various proteins are required to different extents for ribosomal activity. The proteins could be divided into several groups according to the activity of "50 S subunits" reconstituted in their absence: (a) strongly required (3 to 25%), 3, 4, 5, 6, 10, 13, 18, 20b, and 26; (b) moderately required (32 to 58%), 2, 8, 11, 21, 22, 23, and 29; (c) weakly required (65 to 84%), 20a, 24, 26, 34, and 38; (d) not required (91 to 100%), 16, 28, 30, and 32; and (e) not required and sometimes inhibitory, 1 and 37 (data not shown).

*In previous studies, using only total protein extract, the reconstitution of peptidyltransferase activity was as efficient as the reconstitution of other ribosomal activities (7, 16). The reason for this difference between the present study and past work is unknown.
Reconstitution of 50 S Ribosomes from Purified Components

**Table III**

**Ethanol/NH$_4$Cl extraction of 50 S subunits**

The extraction procedure and the assay for polyphenylalanine synthesis are described under "Methods." In this assay, the [$^3$H]phenylalanine had a specific activity of 100 Ci/mmol. Assay mixtures contained 1.17 A$_{260}$ units of 50 S subunits. The GTPase assay was conducted as described under "Methods," except that the final GTP concentration was 0.33 mM. GTPase assay mixtures contained 0.2 A$_{260}$ unit of 50 S subunits.

| Protein | Poly(Ph) | GTPase |
|---------|---------|--------|
| pmol    | pmol %  | pmol   | pmol %  |
| 50 S subunits | 5440   | 50%  | 3670   | 2.61% |
| Extracted 50 S subunits | 640    | 12%   | 39     | 0.04% |
| Extracted 50 S subunits + Protein 13 | 5250   | 96%   | 3490   | 2.49% |

**Table IV**

**Activities of 50 S subunits reconstituted in absence of single proteins in polyphenylalanine synthesis**

Reconstitution mixtures contained 0.5 A$_{260}$ unit of 23 S RNA, 0.03 A$_{260}$ unit of 5 S RNA, and a mixture of 1 A$_{260}$ equivalent of all the purified proteins except the one indicated, in 180 µl. In addition, Proteins 1 and 37 were omitted in all experiments. Duplicate 75 µl aliquots of each reconstitution mixture were assayed as described under "Methods" except that the [$^3$H]phenylalanine had a specific activity of 100 Ci/mmol. The activity of each particle is expressed as a percentage of the activity of control particles reconstituted from a mixture containing 20 S RNA, 5 S RNA, and all the purified proteins except Proteins 1 and 37. Each figure represents an average of results from two to five independent experiments, as indicated. In a typical experiment, an equivalent amount of 50 S subunits polymerized 81.5 pmol of phenylalanine (12,168 cpm), and control-reconstituted particles polymerized 34.1 pmol of phenylalanine (42% reconstitution efficiency).

| Omitted protein | Percentage of activity | Number of experiments | Omitted protein | Percentage of activity | Number of experiments |
|-----------------|-----------------------|----------------------|-----------------|-----------------------|----------------------|
| 2               | 14 ± 6                | 5                    | 21              | 58 ± 14               | 3                    |
| 3               | 3 ± 1                 | 2                    | 22              | 37 ± 5                | 3                    |
| 4               | 8 ± 5                 | 3                    | 23              | 46 ± 0                | 3                    |
| 6               | 24 ± 2                | 4                    | 24              | 84 ± 2                | 2                    |
| 11              | 11 ± 3                | 3                    | 25              | 3 ± 0                 | 2                    |
| 8               | 32 ± 8                | 5                    | 26              | 80 ± 11               | 4                    |
| 10              | 18 ± 7                | 3                    | 28              | 68 ± 12               | 3                    |
| 11              | 55 ± 10               | 5                    | 29              | 38 ± 6                | 3                    |
| 13              | 8 ± 4                 | 3                    | 30              | 91 ± 11               | 3                    |
| 16              | 100 ± 11              | 3                    | 32              | 97 ± 5                | 2                    |
| 18              | 25 ± 1                | 3                    | 34              | 76 ± 2                | 2                    |
| 20a             | 6/2 ± 2               | 3                    | 38              | 62 ± 3                | 2                    |
| 20b             | 6 ± 3                 | 3                    |                  |                       |                      |

**Discussion**

**Number of 50 S Subunit Proteins—** This paper describes the resolution of the protein fraction of *B. stearothermophilus* 50 S subunits into its individual components and the reconstitution of these individual components, along with RNAs, into active 50 S subunits. According to the purification procedure employed here, there are 27 proteins in the *B. stearothermophilus* 50 S subunit, or, counting Protein 13 as two proteins (see below), 28 proteins.

Although the original numbering system designated 38 spots on the two-dimensional gel pattern, some of the spots originally numbered are very faint spots which have not been observed consistently, and in certain cases, multiple spots are probably derivatives of a single protein. These spots are as follows.

**Spot 7**—The original pattern showed a faint spot, 7, to the left of Spot 8. This spot has not been observed in the present study. The fainter spot to the right of Spot 8 is seen only when the first dimension gel contains 4% acrylamide and, therefore, was not numbered in the original nomenclature. The purified Protein 8 preparation contains a minor amount of this spot in addition to Spot 8. No column chromatographic procedure that we employed succeeded in separating Protein 8 into two components, and a single band was observed in both the "standard" urea gel system and on sodium dodecyl sulfate gels.

Treatment of total 50 S proteins with performic acid resulted in the disappearance of the double spot pattern and the appearance of a single spot which appeared to be at the same position as Spot 6. Therefore, we regard these two spots, Spot 6 and the right hand spot, as oxidized and reduced derivatives of a single protein. Protein 8. In this connection, we note that the *E. coli* Proteins L8 and L9, which have electrophoretic mobility similar to that of *B. stearothermophilus* Protein 8, also resolve only in 4% gels and have identical molecular weights. Furthermore, both Mora et al. (45) and Zimmerman and Stöffler isolated only a single protein corresponding to *E. coli* L8 and L9.

**Spots 12, 14, and 15**—The spots labeled 13 in Fig. 1a vary greatly in their relative intensities from one sample to another. As mentioned above, all these spots are extracted from ribosomes by the ethanol/NH$_4$Cl procedure used for extracting *E. coli* L7/L12. The proteins in the right hand and left hand spots chromatographed together on all columns that we employed. As in the case of Protein 8, treatment of total 50 S proteins with performic acid resulted in the disappearance of the right hand spots and increased intensity of the left hand spots. Hence, as with Protein 8, it appears that the right and left hand spots are reduced and oxidized derivatives of each other.

In many samples, both the right and left hand regions for Protein 13 appear as a pair of spots vertically separated by a small distance (see Fig. 1b, Fig. 14). Although the gel of Protein 13, shown in Fig. 10, containing 14% acrylamide at pH 4.6, shows a single band, either a double band or a smeared band was often observed for Protein 13 in this gel system (cf. Fig. 3c). Visentin et al. (46) have shown that the "A protein" extracted from *B. stearothermophilus* 50 S subunits resolves into two components on DEAE-cellulose chromatography, A$_1$ and A$_2$, both of which cross-react immunologically with *E. coli* L12. Preliminary results indicate that the A$_1$ protein may have a blocked NH$_2$ terminus. They designated the left hand region of L13 as the electrophoretic position of this protein on two-dimensional gels, and presumably their ribosomes contained no stained material at the right hand position for Protein 13. Thus it appears that Protein 13, like *E. coli* L7/L12, is present in two forms, one with a blocking group at the NH$_2$ terminus, and the degree of blocking varies greatly with growth conditions (46). A single attempt of ours at separating Protein 13 into two forms on DEAE-cellulose was not successful, but this may be largely because the 50 S subunits used for the preparation of Protein 13 contained only small amounts of the upper spots (presumably the form of the protein with the

*J. Cohlberg and M. Nomura, unpublished experiments.

R. Zimmerman and G. Stöffler, unpublished experiments (See Ref. 2).
Reconstitution of 50 S Ribosomes from Purified Components

...through more detailed protein chemistry, such as peptide mapping, end-group analysis, and amino acid sequence studies, and we hope to acquire such information in the future.

If the molecular weights of the \textit{B. stearothermophilus} proteins as determined by sodium dodecyl sulfate gel electrophoresis (Table I) are added together, excluding Protein 1 but counting Protein 13 twice, the sum is \(4.8 \times 10^5\) daltons. This number can be compared to the estimate of \(4.5 \times 10^5\) daltons for the total mass of the protein moiety of 50 S subunits derived from salt-washed ribosomes, based on studies with \textit{E. coli} (48) as well as \textit{B. stearothermophilus}.\(^*\)

Kalschmidt and Wittmann (23) identified 34 spots for \textit{E. coli} 50 S subunit proteins on two-dimensional polyacrylamide gels. One of these, L26, subsequently proved to be identical with a protein from the 30 S subunit, S20 (2). Thus, there appear to be 33 proteins in the \textit{E. coli} 50 S subunit. However, three of these proteins have not been isolated by other groups who have purified the \textit{E. coli} 50 S subunit proteins (cf. Ref. 2), and no data establishing the uniqueness of all 33 proteins has appeared. We have purified 28 proteins from \textit{B. stearothermophilus} 50 S subunits. One of the \textit{B. stearothermophilus} proteins, Protein 1, is larger than any of the proteins present in the \textit{E. coli} 50 S subunit and is dispensable for ribosomal activity if not inhibitory. Therefore, Protein 1 could be a non-ribosomal protein. Although we have not rigorously established the purity of each of our protein preparations by protein chemical analysis, it seems most likely that the number of proteins in the \textit{B. stearothermophilus} 50 S subunit is less than 33. More experiments are required to establish whether this apparent difference in the number of 50 S proteins between the two bacterial species is real or is a result of human error in the determination of the number of proteins for one or both of the species.

Reconstitution of 50 S Subunits with Purified Proteins

This is the first demonstration of the total reconstitution of 50 S subunits of any species from purified molecular components. The first total reconstitution of 50 S subunits from RNA and proteins employed the \textit{B. stearothermophilus} system (6). Subsequently, Maruta \textit{et al.} (49) reported the total reconstitution of \textit{E. coli} 50 S subunits. Since other laboratories failed to reproduce their experiments (cf. Ref. 50), they published a second report (51) giving further details and explanations for the variability of results with their system. Recently Nierhaus and Dohme (52) reported the total reconstitution of \textit{E. coli} 50 S subunits by a different approach. These studies employed unfractionated mixtures of \textit{E. coli} 50 S subunit proteins. Total reconstitution of active \textit{E. coli} 50 S subunits with the use of purified proteins has not yet been demonstrated.

The reconstitution using all purified components demonstrates that the reconstitution of \textit{B. stearothermophilus} 50 S subunits in \textit{vitro} does not require any components from 30 S subunits or any non-ribosomal factors. The 23 S RNA and 5 S RNA used in the reconstitution experiments were free of 16 S RNA, transfer RNA, or other contaminants, as evidenced by the presence of single bands on polyacrylamide gels (data not shown). For \textit{E. coli} there is genetic evidence that 50 S subunit assembly in \textit{vivo} may be facilitated by 30 S subunits or some components thereof (53). There are additional reports suggesting that a non-ribosomal "maturation factor" may play a role.

\(^*\)S. Mizushima, V. Erdmann, and M. Nomura, unpublished experiments.
in 50 S subunit assembly both in vivo and in vitro (54, 55). Since our protein preparations were not all completely pure, and small amounts of 30 S subunit or non-ribosomal components may have been present in small amounts in reconstitution mixtures, we cannot completely exclude the possibility that small amounts of these impurities, acting catalytically rather than stoichiometrically, are necessary for the reconstitution. We also cannot rule out the possibility that such non-50 S subunit components may play a role in the reconstituted activity of 50 S subunits which is not observed in vitro. However, it is reasonable to conclude that for B. stearothermophilus, active 50 S subunits can be assembled in a system containing only components found in native 50 S subunits, and that the participation of other components is not obligatory.

Further indications that the purified system does not lack any important component come from examining the properties of the reconstituted 50 S subunits. A stoichiometric mixture of the purified proteins was slightly more efficient than total protein extracts of 50 S subunits in the reconstitution of active 50 S subunits upon incubation with 23 S RNA and 5 S RNA under reconstitution conditions. The reconstituted 50 S subunits were active in poly(U)-dependent polyphenylalanine synthesis, association with 30 S subunits (Phe tRNA protection assay), EF-G-dependent GTPase, peptidyltransferase, and EF-T-dependent GTPase, although the reconstitution of peptidyltransferase activity was somewhat less efficient than the other ribosomal activities which were examined. The rate of reconstitution was faster with the mixture of purified proteins than with the total protein extract. Most of the reconstituted "50 S subunits" sedimented at the same rate as native 50 S subunits and had a similar protein composition. These experiments demonstrate that our mixture of purified proteins contains all the components necessary for 50 S subunit activity and for efficient reconstitution of 50 S subunits very similar in physical, chemical, and functional properties to native 50 S subunits.

It should be pointed out that none of the assays used to test the functional activities of 50 S subunits, including polyphenylalanine synthesis, requires the polypeptide chain termination function, and a defect in termination activity would not have been detected in our experiments. However, our present understanding of the termination function suggests that it is performed by the same components of the ribosome which are involved in the peptidyltransferase reaction (56, 57). Hence the demonstration that reconstituted 50 S subunits are active in the peptidyltransferase reaction probably indicates that they are also active in chain termination.

**Roles of Individual 50 S Subunit Proteins in Protein Synthesis**—The reconstitution system demonstrated in this report should be useful in studying the roles of the individual proteins in the structure and function of 50 S subunits. To demonstrate the usefulness of the system and to initiate studies on the roles of the individual components in protein synthesis, we have determined the activities of "50 S subunits" reconstituted in the absence of individual proteins in poly(U)-dependent polyphenylalanine synthesis. The results show that in most cases the resultant particles were less active than particles reconstituted from mixtures containing all the purified proteins. Such a decrease in activity indicates that the omitted protein: (a) is essential for 50 S subunit assembly, (b) is directly involved in a 50 S function, (c) is indirectly involved in ribosome function by maintaining an active center of the ribosome in an active conformation, or (d) is required for both assembly and function of 50 S subunits (cf. Ref. 4).

Also, there are several proteins whose omission from reconstitution mixtures has virtually no effect on the activity of the reconstituted particles. This does not necessarily mean that these proteins play no role in the reconstruction or activity of 50 S subunits. For example, the E. coli 30 S protein S16 is not required for ribosomal activity, but the reconstitution reaction proceeds more slowly in its absence (40). The protein S18 is not required for activity when reconstituted particles are assayed directly, but when the reconstituted particles are isolated before assay, their activity is lower because Proteins S11 and S21, which require the presence of S18 for stable binding, are lost during the isolation procedure (40). The proteins which were not required for activity in the experiments reported in Table III might be of the type represented by E. coli S16 or S18. Alternatively, these proteins might be required for some other ribosome functions which are not involved in poly(U)-dependent polyphenylalanine synthesis.

In order to test the specific roles of individual proteins, it will be necessary to prepare 50 S subunits reconstituted in the absence of single proteins, to measure their sedimentation coefficients, determine their protein and RNA compositions, and measure their activity not only in overall polyphenylalanine synthesis but in each of the partial reactions of polypeptide synthesis, such as peptidyltransferase or EF-G-dependent GTPase. Such experiments are now in progress. Also, it should be possible to correlate the B. stearothermophilus proteins with their more extensively studied E. coli counterparts by a combination of substitution experiments and immunological techniques, as was done for the 30 S subunit proteins (34). In short, the reconstitution of B. stearothermophilus 50 S subunits from purified molecular components should prove to be a useful technique for studying the structure and function of 50 S ribosomal subunits.

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Reconstitution of Bacillus stearothermophilus 50 S ribosomal subunits from purified molecular components.

J A Kohlberg and M Nomura

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