Reconstitution of Escherichia coli 30 S Ribosomal Subunits from Purified Molecular Components*

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SUMMARY

Reconstitution of 30 S ribosomal subunits from 16 S RNA and a mixture of purified individual 30 S ribosomal proteins has been studied.

Proteins from the 30 S ribosomal subunit of Escherichia coli were purified by a combination of phosphocellulose and DEAE-chromatography, and Sephadex gel filtration. The proteins purified correspond to the 21 proteins generally accepted as 30 S proteins, with the exception of two proteins, P3b and P3c, which correspond to the protein S6 studied by other workers. P3b and P3c are closely related, and one is probably a derivative of the other.

Using a mixture of these purified proteins, reconstitution of functionally active 30 S subunits has been demonstrated. Reconstituted particles had higher activities in poly(U)-directed polyphenylalanine synthesis than reference 30 S particles in several experiments. The functional activity of reconstituted particles was also examined in several other assays; these included natural messenger RNA-directed polypeptide synthesis, poly(U)-directed Phe-tRNA binding, AUG-directed fMet-puromycin formation, AUG-directed fMet-tRNA binding, and the binding of termination codon UAA in the presence of chain termination factors. In all cases, activities comparable to reference 30 S subunits were observed. The sedimentation properties and the protein composition of reconstituted particles were also similar to 30 S ribosomal subunits. The kinetics of reconstitution using purified protein mixtures was essentially identical with those of reconstitution using unfractionated 30 S proteins. These results strongly suggest that 21 purified 30 S proteins together with 16 S RNA are sufficient to reconstitute 30 S subunits, and that no essential 30 S components were lost during the fractionation and purification of the 30 S proteins.

Single component omission experiments indicated that all purified proteins, except P1(S1) and P3b,c(S6), are required for full functional activity. A requirement for P9a(S16) has been shown to be involved in the reconstitution reaction in other experiments (Mizushima, S., and Nomura, M. (1970) Nature 226, 1214; Nomura, M. (1973) Science 179, 864) and therefore are 30 S components.

It is still not clear whether P1(S1) should be considered a "true" 30 S protein or a ribosomal-associated "factor."

The 30 S ribosomal subunit of Escherichia coli consists of one 16 S RNA molecule and about 20 protein molecules. The 30 S subunit consists of one 23 S RNA molecule, one 5 S RNA molecule, and about 30 to 35 protein molecules (for reviews, see 1–4). We have previously shown that bacterial 30 S subunits can be reconstituted from their dissociated molecular components (5). This indicates that the information for the correct assembly of ribosomal particles is contained in the structure of their molecular components, and not in any non-ribosomal factors. In addition, the reconstitution system has established a method of analyzing functions of individual molecular components (for reviews, see 1 and 6).

Our initial reconstitution studies were done using purified 16 S RNA and a 30 S ribosomal protein mixture extracted from purified 30 S subunits. In order to accomplish unambiguous identification and functional analysis of all the essential molecular components of the 30 S subunit, it is necessary to separate and purify each of the proteins contained in the 30 S subunits and then to show complete reconstitution of functionally active particles from the 16 S RNA and a mixture of each of the purified proteins. For this purpose, we separated and purified 30 S ribosomal proteins (7–9), as did other investigators (9–13). We have shown that functionally active 30 S subunits can be reconstituted from a mixture of these purified molecular components. Preliminary reports of these experiments have already been published (7, 14, 15). In this paper, we describe in detail our method of purification of each of the 30 S ribosomal proteins, as well as characterization of the 30 S ribosomal subunits reconstituted from the pure protein components and the 16 S RNA molecule.

MATERIALS AND METHODS

Buffers—Buffer I: 10 mM Tris·HCl, pH 7.4 (24°), 30 mM NH₄Cl, 10 mM MgCl₂, 6 mM 2-mercaptoethanol. Buffer II: same as Buffer I except 0.3 M MgCl₂. Buffer III: 6 M urea, 10 mM phosphoric acid, pH adjusted to 8.0 with methylamine, and 3 mM 2-mercaptoethanol. Buffer IV: 30 mM Tris·HCl, pH 7.4 (24°), 20 mM MgCl₂, 6 mM 2-mercaptoethanol. Buffer

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Two nomenclatures have been made previously (9). This will be handled differently in this paper. Used of hart are employed in zonal centrifugation (Ickman, '15 rotor) centrifugation has been described (16). More recently, we have used two methods to obtain cell-free extracts. The "glass bead" method has been described (16). More recently we have found that better results are obtained using the alumina-grinding method (17). Cell paste, 100 g, was ground in a large mortar with 200 g of alumina (Fisher) at 4°C. The alumina and broken cells were suspended in about 300 ml of Buffer I. DNase (Worthington, RNase-free) was added to about 2 μg per ml, the suspension incubated at 4°C for 20 min, and then centrifuged at 15,000 rpm for 20 min (Sorvall centrifuge, SS34 rotor). The supernatant was saved, the alumina resuspended in about 200 ml of Buffer I, and centrifuged again. The two supernatants were combined and centrifuged at 37,000 rpm for 30 min (Beckman 30 rotor) to remove some traces of alumina and cell debris. The crude extract thus obtained was centrifuged at 35,000 rpm for 12 hours (Beckman 35 rotor) to sediment the ribosomes. The pellet was suspended in 60 to 100 ml of Buffer I and stored frozen at ~70°C in 100-g amounts.

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We have found that dialysis of TP30 against Buffer III results frequently in precipitation of some proteins. PI2(818), for example, is not very soluble in 6 M urea buffers unless some LiCl (or other suitable salts) is present. Therefore, the phosphocellulose column fractionation at pH 8.0 was done in two steps. The first phosphocellulose column (PC-1, Fig. 1) consisted of a gradient of 0.15 to 0.6 M LiCl in Buffer III (pH 8.0). The pass-through fraction was then subjected to the second phosphocellulose column chromatography (PC-2, Fig. 1).

Mamnuex P standard capacity phosphocellulose (0.9 maq per g, Schwarz-Mann) was washed several times with water to remove fine particles. It was then treated with 0.1 n NaOH at room temperature for 10 to 15 min, filtered, and washed with water until the pH was about 8.0. The phosphocellulose was then suspended in water, methylamine added (3 ml per liter), and the pH adjusted to 8.0 (or 6.5 when indicated) with concentrated phosphoric acid. The phosphocellulose was again filtered and washed three times with the starting buffer (0.15 M LiCl in Buffer III). The slurry was degressed under vacuum at room temperature for 20 to 30 min before pouring. The column (2.5 x 90 cm), packed fairly tightly with the phosphocellulose, was then equilibrated with starting buffer (0.15 M LiCl in Buffer III) at 4°C until the pH of the effluent was the same as the buffer (pH 8.0).

The sample, containing about 1 g of ribosomal protein (from 50,000 A 260 units of 30 S ribosomes), was dialyzed, first against approximately 13 volumes of Buffer III overnight to reduce the LiCl concentration to approximately 0.15 M. The sample was then dialyzed for 6 to 8 hours against 10 to 20 volumes of starting buffer before it was applied to the column. After the column was washed with approximately 400 ml of the same buffer, the

1 Nomenclature of 30 S ribosomal proteins in this paper is that used in our own laboratory (1, 8, 36). The standard nomenclature adopted (9) is shown in parentheses. The correlation of the two nomenclatures has been made previously (9). This will be further discussed in this paper.
the methods used are described in the text and the footnote to S6 (9). See the text for discussion. The elution pattern of P13(S19) also varied with starting buffer. When protein fractions were to be applied to a Sephadex column, pertinent phosphocellulose column fractions P14(S20) and P15(S21) by a phosphocellulose column using a gradient of 0.4 to 0.7 M LiCl in Buffer III, pH 6.5. P14(S20) and P15(S21) were separated using the same conditions (PC-5, Fig. 1). The elution pattern of PC-1 has occurred in 1260 units of 30 S ribosomes) were combined before further purification. Slight variation in the elution pattern of PC-1 has occurred with different batches of phosphocellulose. For example, Pl0(S12) has not always been clearly separated from P14(S20).

| Protein | Contaminating proteins present after PC-1 | Further purification steps |
|---------|-----------------------------------------|-----------------------|
| P1(S1)  | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P2(S2)  | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P3(S3)  | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P3a     | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P3b(S6) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P3c(S6) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P4(S5)  | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P4a(S4) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P4b(S8) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P5(S7)  | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P6(S10) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P7(S11) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P8(S9)  | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P9a(S16) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P10(S12) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P10a(S13) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P10b(S15) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P11(S14) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P12(S18) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P13(S19) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P14(S20) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |
| P15(S21) | Pl, P2(S2), P4(S5), P3b and P3c(S6), P6(S10), and P3a, was dialyzed against Buffer III (pH 8.0). | Pl-DEAE, G-100 |

* Abbreviations and chromatography conditions: PC (phosphocellulose), DEAE (DEAE-cellulose), G-75, or G-100 (Sephadex G-75 or G-100). PC-1: column (2.5 X 90 cm), pH 8.0, 0.15 to 0.6 M LiCl, 8.0 liters. PC-2: column (2.5 X 90 cm), pH 8.0, 0 to 0.25 M LiCl, 6.0 liters. PC-3: column (1.8 X 30 cm), pH 8.0, 0.1 to 0.3 M LiCl, 2.0 liters. PC-4: column (1.8 X 40 cm), pH 8.0, 0.25 to 0.45 M NaCl, 3.0 liters. PC-5: column (1.8 X 30 cm), pH 8.0, 0.4 to 0.7 M LiCl, 2.0 liters. DEAE: column (2.5 X 90 cm), pH 8.0, 0 to 0.15 M LiCl, 6.0 liters. G-75, G-100: column (2.5 X 90 cm), elution buffer, 0.15 M LiCl in Buffer III, pH 6.5.

The pass-through fraction from PC-1 contains P1(S1), P2(S2), P3a, P3b(S6), P3c(S6), P4(S5), P4b(S8), and P6(S10).

Step not always required.

were combined and then concentrated to about 5.0 ml. This was accomplished by first diluting the phosphocellulose column fractions with 3 volumes of Buffer III (pH 8.0) to lower the salt concentration and then applying this sample to a small phosphocellulose column (1.5 X 7 cm). The protein was then eluted with 2 M LiCl in Buffer III. Two to three liters of diluted protein sample were thus concentrated to about 10 to 20 ml. For further concentration, the sample was placed in a dialysis membrane and covered with dry Sephadex G-200 or sucrose. Sephadex columns (2.5 X 90 cm) were equilibrated with 0.15 M LiCl in Buffer III (pH 6.5) and eluted with the same buffer at a flow rate of 8 to 10 ml per hour. Fractions of 3.0 to 4.0 ml were collected.

Table I summarizes our purification methods and Fig. 1 shows an example of results obtained by these methods.

Slight variation in the elution pattern of PC-1 has occurred with different batches of phosphocellulose. For example, P10(S12) has not always been clearly separated from P14(S20) and P15(S21). In such cases, P10(S12) was separated from P14(S20) and P15(S21) by a phosphocellulose column using a gradient of 0.4 to 0.7 M LiCl in Buffer III, pH 6.5. P14(S20) and P15(S21) were separated using the same conditions (PC-5, Fig. 1). The elution pattern of P13(S19) also varied with

30 S proteins were eluted with a linear gradient of 0.15 to 0.8 M LiCl in Buffer III (total volume, 8.8 liters), pH 8.0, at a flow rate of 80 to 90 ml per hour. Fractions of 15 ml were collected and analyzed for protein by absorption at 230 nm (PC-I, Fig. P3a, P3b(S6), P3c(S6), P4(S5), P4b(S8), and P6(S10).

Protein fractions from two PC-I columns (i.e. protein from about 100,000 $A_{260}$ units of 30 S ribosomes) were combined before further purification. Column fractions to be applied to another phosphocellulose column were suitably combined and dialyzed against the next starting buffer. When protein fractions were to be applied to a Sephadex column, pertinent phosphocellulose column fractions

Fig. 1. Purification of 30 S ribosomal proteins. The details of the methods used are described in the text and the footnote to Table I.
different batches of phosphocellulose. Pi3(S19) sometimes eluted with Pi1(S14) rather than Pi0a(S13) as shown in Fig. 1 (PC-1). Pi1(S14) and Pi3(S19) were separated (PC-4, Fig. 1) with phosphocellulose at pH 6.5 using a gradient of NaCl from 0.25 to 0.45 M (similar to the method used by Hardy et al. (10)). The separation of Pi0a(S13) and Pi3(S19) was not complete under these conditions so Sephadex chromatography was used when Pi3(S19) and Pi0a(S13) eluted together (Fig. 1). Since the separation of Pi9a(S16) and Pi9b(S17) was not complete after the first phosphocellulose chromatography (PC-1, Fig. 1), a second chromatography was necessary on phosphocellulose at pH 6.5 using a 0.1 to 0.3 M LiCl gradient (PC-3, Fig. 1). In our earlier report (7), we failed to separate Pi9a(S16) from Pi9b(S17) and incorrectly regarded a mixture of the two proteins as one protein called Pi9.

During the various purification steps, the amount of contaminating proteins was usually determined by gel electrophoresis of column fractions. In some cases, we have used immunochemical methods (15, 23) to determine cross-contamination. For example, only those Pi9a(S17) fractions free of Pi9b(S16), as judged immunochemically, were pooled.

As a final purification step, most phosphocellulose or DEAE-fractions were applied to a Sephadex G-75 or G-100 column (see Table I, also Fig. 1).

The final recovery of a purified protein (mole of pure protein per mole of starting 30 S subunits) may depend on several factors, such as the number of steps required to obtain the pure protein, and whether the protein is found in "fractional" or "unit" amounts (24) in isolated 30 S subunits. In the best cases, Pi4(S4), Pi4(S4), Pi8(S8), Pi9a(S16), and Pi8 plus Pi10c(S0) were obtained in 40 to 50% yields; Pi5(S5), Pi7(S7), Pi11(S14), Pi12(S20), and Pi9b(S17) in 20 to 40% yields; Pi3(S3), Pi10(S10), Pi10(S10), Pi11(S11), Pi10(S10), Pi10(S10), Pi12(S18), and Pi13(S19) in 20 to 30% yields; and Pi1(S1), Pi2(S2), Pi10(S10), and Pi15(S21) in 15 to 20% yields.

Solubility and Storage of Proteins—As mentioned previously, some 30 S proteins are rather insoluble, even in 6 M urea buffers, unless some salt is present. Therefore, we store purified proteins (frozen at −70 °) in Buffer III containing 0.15 M LiCl. The proteins appear to be stable for a year or more under these conditions in regard to their ability to be reconstituted into active 30 S particles. Most of the proteins are soluble in Buffer V or Buffer VI (except Pi12(S18)) but may precipitate after repeated freezing and thawing (especially Pi12(S18), Pi6(S10), and Pi5(S7)). To obtain solutions of these less soluble proteins in Buffer VI, concentrated stock solutions (in Buffer III containing 0.15 M LiCl) are diluted about 10-fold into Buffer VI and dialyzed for a short period of time (1 hour). (For reconstitution, these proteins are further diluted [at least another 10-fold]. Presence of small amounts of urea does not appear to interfere with reconstitution.) Although we have not made detailed studies, some proteins (Pi6(S10), Pi3(S3), Pi2(S2), Pi7(S11), and Pi12(S18)) appear to lose some "functional" activity after repeated freezing and thawing (especially in nonurea buffers). Mixtures of purified proteins appear to be more soluble and stable than individual proteins.

Reconstitution Procedure—Reconstitution from a mixture of purified proteins and 16 S RNA was done by adding approximately 2 moles of each purified protein to each 16 S RNA. The molecular weight of 16 S RNA was considered to be 550,000 (25, 26), and the average molecular weight values for each of the purified proteins reported by Dzionara et al. (27) were used for the calculation. Since 1 A260 unit of 16 S RNA corresponds to 80 pmole, we define 80 pmole of each of the pure proteins as 1 A260 eq. The purified proteins were mixed at the appropriate concentration and dialyzed against Buffer VI. Individual proteins needed for titration experiments were diluted from concentrated solutions to 150 A260 eq per ml and dialyzed against Buffer VI. As mentioned above, some proteins (Pi5(S7), Pi6(S10), and Pi12(S18)), were dialyzed for only 1 hour. Phenol-extracted 16 S RNA (16) was dialyzed against Buffer IV.

RNA and proteins were mixed in the molar ratio of 1:1.8 unless otherwise specified. The volume ratio of Buffer IV to Buffer VI in the final reconstitution mixture was 1:2, giving an optimal KCl concentration for reconstitution of 0.33 M (28). The reconstitution mixture, containing between 3.6 and 10 A260 units of 16 S RNA per ml, was then heated for 60 min at 40° (or as indicated). The mixture was cooled on ice, and reconstituted particles were isolated by centrifugation in the reconstitution buffer ("isolated particles"). Alternatively, the particles were purified by sucrose gradient centrifugation (9 to 20% in Buffer VII); the peak fractions were pooled and particles were recovered by centrifugation ("purified particles"). The particles were suspended in Buffer I, and their functional activities determined (see below).

'Direct Assay' of Activity of Reconstituted Particles—In several experiments, aliquots (up to 50 μl containing 0.15 to 0.25 A260 unit of reconstituted particles) were taken from the reconstitution mixtures, and the activity of the reconstituted particles in poly(C) directed [35S]phospholipase incorporation (16) was assayed directly without prior isolation of the reconstituted particles. In this case, the assay method described previously (16) was modified slightly so that the amount of reconstitution during the assay could be minimized. This was accomplished by increasing the specific activity of the [35S]phospholipase from 10 to 50 μCi per pmole and reducing the incubation time and temperature to 10 min at 30°. The total volume of the assay mixture was 200 μl and the amount of all other assay components was the same as described previously (16). The extent of reconstitution during assay using these conditions was less than 10% of that obtained under standard reconstitution conditions (10°, 60 min in Buffer VII). With this direct assay method, reconstitutions using as little as 0.15 A260 unit of 16 S RNA have been possible.

Other Methods—The functional assays of purified reconstituted particles for poly(U)-directed [35S]phospholipase in incorporation, 2 RNA-directed [3H]valine incorporation, poly(U)-directed [3H]phenylalanine-TRNA binding, and formation of fMet-purumycin have been described previously (16, 29). The binding of [3H]UAPNA in the presence of release factors RF-1 or RF-2 was done essentially as described by Scolnick and Caskey (30) (see also 31). The assay for binding of fMet-tRNAmet to 30 S ribosomes in the presence of purified initiation factors IF2 or IF-2 + IF-1 contained the following in a final volume of 50 μl: 30 mM Tris-HCl, pH 7.4 at 24°; 100 mM NH4Cl; 5 mM magnesium acetate; 1 mM diethiothreitol; 0.03 A260 unit of ApEpG; 1 mM GTP; approximately 1.5 × 10⁶ cpm of [3S]Met-tRNAmet (purified tRNAmet obtained from Oak Ridge National Laboratories was charged with [3H]methionine, 100 Ci per mm obtained from New England Nuclear Corp., 39; 38); 0.05 A260 unit of 30 S ribosomes or reconstituted particles; 1 μg of IF-2 (and where indicated 0.1 μg of IF-1). The reaction mixture was incubated at 30° for 5 min, terminated by addition of 1.0 ml of cold buffer (10 mM Tris-HCl, pH 7.4 at 24°; 10 mM magnesium acetate; 50 mM NH4Cl), and filtered slowly through Millipore filters (HAWP 2400) followed by washing with the same buffer
three times. The filters were then dried and counted in a liquid scintillation counter with a toluene-based scintillation fluid. Purified initiation Factors IF-1 and IF-2 were generously supplied by Dr. J. W. Hershey (cf. 34). All assays were tested and found to be linear with respect to ribosome concentrations used. Reference 30 S subunits were preheated at 40° for 20 min in Buffer I before assay. Preincubation in Buffer VII (reconstitution buffer) gave similar activities.

One-dimensional polyacrylamide electrophoresis was done at pH 4.5 as described previously (16). Two-dimensional polyacrylamide electrophoresis of ribosomal proteins was done according to the method of Kaltschmidt and Wittmann (19). Protein concentration was measured by the method of Lowry et al. (35), using crystalline bovine serum albumin as a standard. The color yield of the bovine serum albumin in this reaction was compared with that of TP30, purified 30 S ribosomal proteins P4a(S5) and P4b(S8). At protein concentrations where usual assays were done, TP30 and P4a(S5) gave the same color yield as bovine serum albumin, whereas P4b(S8) gave a color yield about 7% less than bovine serum albumin.

RESULTS

Purified 30 S Ribosomal Proteins Used for Reconstitution—As described under "Materials and Methods," we have purified 23 proteins from 30 S subunits (Fig. 1 and Table I). The purity of these proteins was examined by one-dimensional polyacrylamide electrophoresis at pH 4.5 (10% polyacrylamide). As shown in Fig. 2(a), most of the proteins showed a single major band. Those proteins which migrate together under the standard conditions were then examined for possible cross-contamination using other methods. For example, P3(S3), P3b(S6), and P3c(S6) migrate together in the standard gel electrophoresis at pH 4.5 (7, 16), but are separable in 8% acrylamide at pH 8.6. (The latter are the conditions used in the first dimension in the two-dimensional electrophoresis (19)). As can be seen in Fig. 2(c), Preparations P3(S3), P3b(S6), and P3c(S6) were free from

![Fig. 2. Polyacrylamide gel electrophoresis of purified 30 S ribosomal proteins. (a) 10% gels, pH 4.5, approximately 15μg of each of the purified proteins were applied to each gel. (b) 4.4% gels, pH 4.5, approximately 7.5μg of each protein were applied to each gel. The migration of proteins was from the center of gels. The condition is identical with that used for the first dimension in two-dimensional gel electrophoresis. Anode is at the left, and cathode is at the right.](http://www.jbc.org/)

![Fig. 3. Two-dimensional electrophoresis of P3b(S16), P3b(S17), P10(S12), P10(S13), and P10b(S15). (a) Two-dimensional polyacrylamide electrophoresis was done according to the method of Kaltschmidt and Wittmann (19). Approximately 100μg of protein were applied to each gel. (b) Electrophoresis of oxidized and reduced forms of P3b(S17) and P10(S12) in 18% polyacrylamide gels. Disc gel electrophoresis (18% polyacrylamide in siliconized glass tubes, 0.6 × 11 cm) was done using conditions identical with the second dimension of the two-dimensional gel method of Kaltschmidt and Wittmann (19). Approximately 15μg of each protein were applied to each gel. Samples were "oxidized" by polymerizing the sample gel with ammonium persulfate. Samples were "reduced" by applying the sample in the "sample gel" containing 2% mercaptoethanol. Presence of mercaptoethanol prevented polymerization of the "sample gel." Electrophoresis was done at room temperature with a current of 2 ma per tube for 8 hours.](http://www.jbc.org/)
Each other. Separation of P3b(S6) and P3c(S6) is consistent with the results obtained with DEAE-column chromatography (see above and Fig. 1). P4(S5), P4a(S4), and P4b(S8) also migrate together under the standard one-dimensional electrophoresis, but can be separable in electrophoresis at lower gel concentrations (e.g. 4.4% instead of 10%). As can be seen in Fig. 2(b), no significant cross-contamination was observed among the Preparations P4(S5), P4a(S4), and P4b(S8). Proteins P9a(S16), P9b(S17), P10(S12), P10a(S13), and P10b(S15) also migrate together in the standard one-dimensional electrophoresis. These proteins are separable by two-dimensional electrophoresis (see Fig. 7(b)), although separation of P9a(S16) from P9b(S17) is not always complete. These protein preparations were therefore examined individually by two-dimensional electrophoresis. They were shown to be free from each other, except that the degree of cross-contamination of P9a(S16) and P9b(S17) was difficult to assess by this method (Fig. 3(a)). It should be noted here that both P10(S12) and P10b(S17) usually give rise to spots in two-dimensional electrophoresis. Electrophoresis of P10(S12) and P9b(S17) under “oxidized” and “reduced” conditions shown in Fig. 3(b) indicates the faster moving spot of P10(S12) and P9b(S17) under “oxidized” and “reduced” conditions under “oxidized” and “reduced” conditions in two-dimensional electrophoresis. The position of P10(S12) in two-dimensional electrophoresis, but its mobility in one-dimensional electrophoresis is about the same as P3a(S3) and is distinguishable from P4(S5) (data not shown, see also 7). The omission of P3a during reconstitution, however, has no significant effect on the activity of the reconstituted particle in several assays (7). P3a, therefore, may not be a true 30 S protein. Although the possibility cannot be excluded that P3a is a ribosomal protein and is important for some function we have not tested, we have omitted this protein from the standard protein mixture for reconstitution experiments described in this paper.

Two proteins (P3b and P3c) corresponding to S6 (Wittmann’s nomenclature11) have been purified in our laboratory from E. coli strains K-12 (see above, and Fig. 2(e)) and MRE600,14 whereas other laboratories have reported only one protein (9). Amino acid sequence analysis of the two proteins from MRE600 indicates that the primary sequences of the first several NH2-terminal residues are identical.1 Also, reconstitution experiments indicate that P3b and P3c are functionally interchangeable (7, 36). Both P3b and P3c correspond to Protein S6 isolated by Wittmann et al.,15 as judged by electrophoretic mobility as well as immunological behavior (9). Possibly, one of these proteins is a derivative of the other. Complete sequence analysis should provide the answer. In the present reconstitution experiments, a mixture of equal amounts of both P3b and P3c was used and treated as a single protein (called P3bc or S6).

Although we have some doubts about the validity of calling P1(S1) a 30 S ribosomal protein (see below), we included P1(S1) in the standard 30 S protein mixture. Thus, omitting P3a and counting P3b and P3c as one protein, we used altogether 21 purified proteins in the reconstitution experiments described in this paper.

Reconstitution with Standard Mixture of Purified Proteins and 16 S RNA—Twenty-one purified proteins were mixed in an equal molar ratio as described under “Materials and Methods” (called the standard purified protein mixture). Various amounts of this standard purified protein mixture were added to a constant amount of 16 S RNA and incubated at 40° for 1 h hour under optimal reconstitution conditions. The particles were then assayed directly for their activity in poly(U)-directed [3H]phenylalanine incorporation. The results are shown in Fig. 4.

It can be seen that maximum activity is reached when the molar ratio of the standard protein mixture to 16 S RNA is about 1.7 to 2.4, indicating some protein or proteins are present in less than the calculated amount. This may be due to errors in our estimate of the amount of some proteins using the Lowry reaction (see “Materials and Methods”). Alternatively, it may be due to selective loss of some proteins during several manipulations including dialysis (adsorption to dialysis tubes or glass walls), partial inactivation of some proteins during purification, or combinations of these possibilities. In all experiments reported here, 1.8 to 2.0 molar equivalents of proteins were used per mole of 16 S RNA.

The general shape of the curve shown in Fig. 4 is similar to that obtained in previous experiments using unfractionated total 30 S proteins (37). In the previous experiments, the results were interpreted to indicate a high degree of cooperativity in the assembly of 30 S particles. However, because of the above-mentioned uncertainty in the exact amount of individual proteins used in the present experiments, it is difficult to make a definite interpretation of the shape of the curve in this case. The high efficiency of reconstitution shown in Fig. 4 suggests that our standard purified protein mixture contains all the essential protein components of the 30 S subunits.

Kinetics of Reconstitution—We have compared the kinetics of reconstitution in the present system with reconstitution using unfractionated 30 S ribosomal proteins (TP30). Fig. 5 shows that the kinetic data of reconstitution at 40 and 30° are essentially the same whether using TP30 or purified 30 S proteins. Near-
EQUIVALENTS OF PROTEIN ADDED

FIG. 4 (Zefl). ELeconstitution of 30 S ribosomes in the presence of 10 S RNA and various amounts of the standard purified protein mixture. Twenty-one purified 30 S proteins were mixed in an equal molar ratio. Various amounts of this standard purified protein mixture were added to 16 S RNA at the indicated molar ratio and reconstitution was performed as described under "Materials and Methods." Duplicate aliquots of reconstituted particles (0.22 A260 unit) were assayed for their activity in poly(U)-directed phenylalanine incorporation using the direct assay method. The 30 S subunits gave 4886 cpm/O.2 A260 unit.

FIG. 5 (center). Kinetics of reconstitution at 40 and 30° using either TP30 or a mixture of purified 30 S proteins. Approximately 1.8 eq of TP30 (O—O) or 1.8 molar equivalents of the standard purified 30 S protein mixture (O—O) were added to 16 S RNA and incubated at 30 or at 40°. Duplicate aliquots (0.21 A260 unit) identical kinetics again suggests that we have not missed any important macromolecular component in the purification of 30 S ribosomal proteins.

In the experiment shown in Fig. 5, the assay of functional activity of reconstituted 30 S particles was done without prior isolation of the particles. This may explain the slight differences in the kinetics of reconstitution using TP30 in the present and previous published experiments (28). The reason for the presence of a slight lag at 30° observed in this experiment is not known.

Properties of Reconstituted Particles—As described above, the particles reconstituted from 16 S RNA and purified individual proteins are active in poly(U)-directed polyphenylalanine synthesis. In the experiments described above, the activity of the reconstituted particles was assayed directly without prior isolation (see "Materials and Methods").

We have also isolated reconstituted particles by pelleting them from the reconstitution mixture ("isolated reconstituted particles") and examined their properties. As can be seen in Table II, the isolated particles showed activity comparable to reference 30 S subunits both in poly(U)-directed polyphenylalanine synthesis and R17 RNA-dependent polypeptide synthesis.

As indicated in Table II, considerable variability has been experienced in the relative activity of the reconstituted particles. To conserve our purified proteins, we used 1.8 to 2.0 molar equivalents of the standard protein mixture which is close to the amount necessary for maximum activity, but not sufficiently in excess (see Fig. 4). Thus, variability in loss of some proteins, as well as their inactivation, as mentioned before, may result in somewhat variable activity. We feel that the experiments with were taken at the indicated times and measured for activity in poly(U)-directed phenylalanine incorporation using the direct assay method. The values are normalized to the activity at 60 min at 40°. For TP30, 100 = 7938 cpm; for the purified protein mixture, 100 = 6333. An equivalent amount of 30 S subunits gave 6088 cpm.

FIG. 6 (right). Sucrose gradient sedimentation analysis of 30 S particles reconstituted from 16 S RNA and purified proteins. 3.6 A260 units of reconstituted particles and a small amount of [14C]uracil-labeled reference 30 S subunits were applied to a 5 to 20% sucrose gradient in Buffer VII. Sedimentation was for 16 hours at 24,500 rpm in a Beckman SW 40 rotor. Fractions were collected and analyzed for absorbance at 260 nm (O—O) and for radioactivity (O—O).

Activity of isolated reconstituted 30 S particles in polypeptide synthesis

Activity of reference 30 S subunits is taken as 100%. Data from eight independent reconstitution experiments are summarized.

| Activity of isolated reconstituted 30 S particles in polypeptide synthesis |
|-----------------------------|-----------------------------|-----------------------------|
| % activity                  | Poly(U)-directed phenylalanine incorporation | R17 RNA directed valine incorporation |
| Range                       | 70-120                      | 63-141                      |
| Average                     | 103                         | 113                         |

lower activity do not reflect the maximum attainable activity, and that 30 S particles with activity higher than the reference 30 S subunits can be attained by reconstitution.

The reconstituted particles were also examined by sucrose gradient sedimentation analysis. As shown in Fig. 6, the sedimentation pattern of the reconstituted particles and the reference radioactive 30 S subunits were virtually identical.

The protein composition of reconstituted particles purified by sucrose gradient centrifugation ("purified reconstituted particles"; see "Materials and Methods") was analyzed by two-dimensional polyacrylamide gel electrophoresis. The results shown in Fig. 7 indicate that all 30 S proteins (except P1(S1)) are present in approximately the same amounts relative to 30 S subunits. However, the exact stoichiometry of each of the proteins in the reconstituted 30 S subunits must await quantitative measurements of these proteins. As noted before (36), P1(S1)
is almost completely absent in the purified reconstituted particles as in 30 S subunits extensively purified by high salt washing.

Various other assays related to 30 S functions were also used to assess the functional activity of the reconstituted particles. Purified reconstituted particles were used in this case. As shown in Table III, the reconstituted particles showed activities comparable to reference 30 S subunits in the fMet-puromycin reaction (in the presence of 50 S subunits), the binding of termination codon UAA (in the presence of 50 S subunits and chain termination Factors RF-1 or RF-2) as well as the binding of Phe-tRNA directed by poly(U) in the presence of 50 S subunits. The binding of fMet-tRNA in the presence of purified initiation factors, IF-1 and IF-2, by the reconstituted particles was somewhat lower (48 to 80%) than the reference 30 S subunits. Since our reference 30 S subunits are not salt-washed, it is possible that some non-ribosomal protein factors which stimulate fMet-tRNA binding (such as IF-1 or IF-3) are present in the reference 30 S subunits. Such factors are not present in the reconstituted particles assembled from purified known components. The lower activity of the reconstituted particles could be explained on this basis. AUG-directed fMet-tRNA binding was also assayed in the presence of IF-2 only. The activity of the reconstituted particles relative to the reference 30 S subunits in this assay was significantly lower than in the presence of both IF-1 and IF-2. These results are consistent with the explanation given above. Either IF-1 or IF-3, in the presence of IF-2, is known to stimulate fMet-tRNA binding (34, 39, 40). It is possible that IF-1 (or IF-3, or both) is present in the reference 30 S subunits in significant amounts.

The data shown in Table III also suggest that purified reconstituted particles are somewhat less active than the isolated (unpurified) reconstituted particles (cf. Table II), both in poly(U)-directed polypeptide synthesis and phage RNA-directed polypeptide synthesis. It is possible that the extra purification steps remove some important proteins or cause inactivation of a small fraction of the reconstituted particles. However, further studies are required to establish this conclusion.

**Single Component Omission Experiments**—The data described so far indicate that 21 purified proteins together with 16 S RNA are sufficient to reconstitute 30 S particles with properties similar to the original reference 30 S subunits. We have then examined the question of whether all of the 21 proteins used are required for reconstitution of functional 30 S particles. For this purpose, single component omission experiments were performed; the reconstitution was performed using a mixture of purified proteins with a single protein omitted. Activity of the reconstituted particles in various functional assays was then analyzed either directly or after isolation and purification of the reconstituted particles. Preliminary experiments of this kind have been described in an earlier paper (7). However, in the earlier experiments, protein-deficient particles were prepared using several protein fractions (with known protein composition) in combination with several purified proteins. This was done in order to conserve purified protein preparations. Moreover, as mentioned before, the uncharacterized Fraction PM containing P10b(S15) was used instead of purified P10b(S15). In addition, a mixture of P9a-

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**Table III**

| Functional activities of purified reconstituted particles | Poly(U)-Phe incorporation | f2 RNA-Val incorporation | fMet-puromycin binding | fMet-tRNA binding | RF1 | RF2 | RF2-IF1 binding | RF2 binding |
|---------------------------------------------------------|---------------------------|--------------------------|-----------------------|------------------|-----|-----|----------------|------------|
| Purified reconstituted particle (P12)                    | 3335                      | 3820                     | 3745                  | 31054            | 60024 | 3344 | 3398           | 2983       |
| 30S                                                     | 3977                      | 4862                     | 3319                  | 61505            | 74723 | 2668 | 3152           | 3094       |
| (% Activity)                                            | (83.9)                    | (72.6)                   | (112.8)               | (50.5)           | (80.3) | (125.3) | (107.8) | (96.4)       |
| % Activity in 3 experiments                              |                           |                           | 69-84                 | 58-73            | 97-113 | 28-51 | 48-80 | 82-192 | 88-175 | 96-112 |
| Average                                                 | 79.4                      | 64.4                     | 70.2                  | 48.3             | 90.2   | 114.0 | 100.8          |        |
TABLE IV

| Protein(s) omitted | Direct assay | Assay after purification | Data in previous system |
|-------------------|--------------|--------------------------|-------------------------|
|                   | Exp. 1 | Exp. 2 | Av. | Exp. 1 | Exp. 2 | Av. |                      |
| frno              | 100   | 100   | 100 | 100   | 100   | 100 |                      |
| P1(S1)            | 89    | 89    | 89  | 101   | 95    | 99  |                      |
| P2(S2)            | 89    | 89    | 89  | 101   | 95    | 99  |                      |
| P3(S3)            | 27    | 20    | 23  | 23    | 23    | 38  |                      |
| P3d5S3c(S16)      | 110   | 110   | 110 | 91    | 96    | 99  |                      |
| P4(S4)            | 30    | 40    | 35  | 41    | 31    | 36  |                      |
| P6(S6)            | 71    | 11    | 16  | 27    | 17    | 23  |                      |
| P6d7(S7)          | 24    | 24    | 24  | 7     | 16    | 13  |                      |
| P7(S7)            | 19    | 19    | 19  | 19    | 19    | 19  |                      |
| P6(16)            | 16    | 9     | 4   | 12    | 8     | 12  |                      |
| P7(22)            | 7     | 7     | 7   | 6     | 6     | 6   |                      |
| P8(S8)            | 57    | 77    | 67  | 48    | 43    | 49  |                      |
| P8d9(S9)          | 91    | 73    | 82  | 103   | 105   | 105 |                      |
| P9(S10)           | 87    | 40    | 63  | 91    | 32    | 62  |                      |
| P9PS8             | 60    | 27    | 46  | 66    | 42    | 59  |                      |
| P10(12)           | 39    | 35    | 43  | 22    | 32    | 29  |                      |
| P10d12(S12)       | 50    | 107   | 78  | 58    | 86    | 68  |                      |
| P10d7(S15)        | 11    | 46    | 29  | 33    | 63    | 48  |                      |
| P11(14)           | 9     | 21    | 14  | 5     | 12    | 6   |                      |
| P12(15)           | 59    | 78    | 69  | 47    | 58    | 53  |                      |
| P13(S19)          | 20    | 30    | 25  | 39    | 38    | 39  |                      |
| P14(S20)          | 06    | 33    | 23  | 22    | 59    | 46  |                      |
| P15(S21)          | 29    | 52    | 41  | 37    | 39    | 39  |                      |
| P8a(S16)          | 92    |       |     |       |       |     |                      |

* Data taken from Ref. 7 (see the text).

(S16) and P9b(S17) was used, and the requirement of each alone was not studied.

We have repeated the same kind of experiments using the 21 purified proteins described above. The results obtained in two independent experiments using different protein preparations are shown in Table IV. In this table, the results on requirements of individual proteins for poly(U)-directed polyphenylalanine synthesis are given. The activity in various other functional assays was also determined in similar experiments, but will be reported in a separate paper.

Two assays were performed in each of the two experiments described in Table IV: (a) direct assay without isolation of particles; and (b) assay after purification of the particles. For comparison, the data shown in the earlier report (7) is also included. Several conclusions can be drawn from the data.

1. All the purified 30 S proteins except for P1(S1), P3b,c(S6), and P9a(S16) are required for maximum functional activity in poly(U)-directed polyphenylalanine synthesis. Although in the two experiments shown in Table IV, a requirement of P9a(S16) was not clearly shown, the requirement was shown in other experiments. P9a(S16) was found to have a special role in the assembly of 30 S particles.6

2. There is a variability in the data among different experiments; for example, the samples having both P9a(S16) and P9b(S17) omitted. As mentioned above, the omission of P9b(S16) alone also gives variable results. We have found that in the absence of both P9a(S16) and P9b(S17) or P9a(S16) alone, the rate of reconstitution is very slow compared to the complete system containing these proteins. The optimum conditions for reconstitution in the absence of these proteins are different from the standard conditions. The activities of these protein-deficient particles relative to control particles can vary considerably depending on the duration of incubation or changes in the conditions of reconstitution. The unique role of these proteins in the assembly reaction will be described separately.6

Another factor may be variability in the degree of inactivation or purity of individual proteins in different protein preparations, or loss of some proteins in the process of making various protein mixtures from individual proteins. Such possibilities have been mentioned before in this paper. It is our experience that more reproducible results were obtained when experiments were repeated using the same protein preparations.

3. Several protein-deficient reconstituted particles (e.g. particles reconstituted in the absence of P12(S18)) showed a high activity when assayed directly, but only a weak activity when assayed after purification of the particles in the same experiment. It is probable that P12(S18), for example, is not directly required for function but is required for the binding of some other proteins. These proteins may be weakly bound to the reconstituted particles in the absence of P12(S18), but lost during purification, resulting in a drastic decrease in the activity of the reconstituted particles. Interdependence of protein bindings during assembly process has been studied, including the role of P12(S18) in the assembly process (36).

Stoichiometry of Individual Proteins Required for Maximum Activity in Reconstitution—Since we now can reconstitute 30 S subunits from 16 S RNA and a mixture of 21 purified proteins, we should be able to determine the amount of each protein needed for maximum reconstitution activity per unit amount of 16 S RNA. For this purpose, preliminary "titration experiments" were done with several proteins. In these experiments, all proteins, except the protein being tested, were added in 2.0 molar equivalents relative to 16 S RNA. Various amounts of the protein to be tested were then added, and reconstitution was performed as shown in Fig. 8. Maximum activity was obtained when approximately 1 eq was added in the case of proteins P4a(S4), P6(S10), P10(S12), and P13(S19). In the case of P7(S11), a slightly higher than 1 eq amount (1.2 to 1.4 eq) was required, whereas somewhat less than 1 eq (about 0.8 eq) of P15(S21) was required for maximum activity. However, these numbers are preliminary. Again, the main uncertainty may come from errors in estimating the amount of individual proteins by the Lowry's reaction using serum albumin as a standard (however, P4a(S4) was shown to give almost the same color yield as serum albumin (see "Materials and Methods")), and the possible loss of proteins during dialysis to remove urea before reconstitution. However, with further experiments, these possible errors could be avoided, and it should be possible to determine accurate stoichiometry for most of the proteins required for reconstitution of active 30 S subunits.

DISCUSSION

Ribosomal proteins from E. coli 30 S subunits have been isolated and purified in several laboratories. Correlation has been made between proteins from different laboratories and 21 proteins, designated as S1 to S21, have been generally accepted as 30 S ribosomal proteins (9).
In this paper, we have first described in detail our own purification method of 30 S ribosomal proteins. Two differences are noted from the generally accepted list of 30 S proteins. First, we have isolated two proteins, P3b and P3c, which correspond to S6. As discussed under “Results,” it appears that one protein is a derivative of the other. The significance of this observation is not clear and must await further chemical studies on these proteins. Second, we have isolated protein P3a which does not correspond to any of the proteins other workers have isolated. As already mentioned, our previous reconstitution experiments failed to reveal any significant role of this protein in reconstitution. Thus, this protein has been omitted from our list of 30 S ribosomal proteins in accordance with other laboratories.

Purity of the purified individual proteins is important for functional analysis of the ribosomal proteins using the present reconstitution system. Purity was judged by polyacrylamide gel electrophoretic analysis using various systems and by immunological methods. As described under “Results,” most of the ribosomal proteins (except possibly P1(S1)) used for the present studies were more than 95% pure. In addition, sequence analysis of the 30 S proteins (15 out of 21 so far analyzed) purified by the present method has shown that the sequence of the first 20 to 40 amino acid residues from the N-terminal is unique in all cases and is different from one another (except P3b and P3c which have identical sequences, see above). No significant cross-contamination has been observed. However, small amounts of contaminating proteins were occasionally observed. For example, the protein P3a(S3) preparation in Fig. 2 shows a weak band corresponding to P5(S7). The contamination was estimated to be about 5%. Since we added 1.8 to 2 moles of each protein per mole of RNA in the single component omission experiments, this contamination would mean that our (-P5(S7)) protein mixtures might have contained up to about 0.1 mole of P5(S7) per mole of 16 S RNA. Thus, some weak residual activity observed with (-P5(S7)) protein mixtures (see Table IV) could be explained, at least partly, on this basis. Nevertheless, purity of the proteins obtained with the present method is satisfactory for most of the experiments designed for functional analysis of these proteins.

The experimental results presented in this paper indicate that the 21 purified proteins together with 16 S RNA molecule are sufficient to reconstitute 30 S particles with functional and physical properties nearly identical with the reference 30 S subunits from which the proteins were isolated. It is important to note in this connection that our reference 30 S subunits are very active as judged by the ratio of poly(U)-directed polyphenylalanine synthesis as well as their activity in AUG-directed fMet-tRNA binding. For example, in the presence of purified IF-1 and IF-2, 3.2 pmoles of the 30 S subunits bound 1.36 pmoles of radioactive fMet-tRNA molecules. Assuming one fMet-tRNA binding site (“initiation site”) per 30 S particle, the data indicate that at least 42% of the 30 S ribosomal particles are in an active state with respect to the initiation function. Since there might be some non-ribosomal stimulatory factors missing in our assay system and the experimental conditions used may not be optimum for the binding, 42% may be a minimum figure. Thus, highly efficient reconstitution relative to the reference 30 S subunits in the present system means that we have probably not missed any ribosomal protein with an important function.

However, this does not necessarily mean that all the 21 proteins used for the reconstitution experiments are “true” ribosomal proteins, especially since several of them are present in amounts less than a single copy per particle in isolated ribosomal preparations (“fractional proteins”) (24, 41). Single component omission experiments have shown that most of the proteins listed are, in fact, required for full activity of ribosomes in various functional assays (the data shown in Table IV and other unpublished experiments, as well as the data published previously (i)). The main exception is P1(S1) and P3b,c(S6).

Voynow and Kurland have shown that P3b,c(S6) are found in about 0.8 copy per isolated 30 S particle (41). Moreover, P3b,c(S6) have a role in the binding of protein P2(S18) during ribosome assembly (36). Therefore, we believe that P3b,c(S6) are 30 S proteins. The situation with P1(S1), however, is different. Omission of P1(S1) from the reconstitution mixture does not cause a reduction in any of the ribosomal functions tested (Table IV and other unpublished experiments; see also (7)). This protein usually fails to get incorporated into the reconstituted ribosome under reconstitution conditions, which include the use of high ionic strength buffers. As shown in the present paper, purified reconstituted particles lack P1(S1) almost completely (see also (36)). Isolated 30 S ribosomal particles contain only about 0.1 to 0.3 copy of this protein per 30 S particle (41). The chemical properties of P1(S1) are also different from all other ribosomal proteins. It is the only protein with a very high molecular weight (mol wt of P1(S1) is 65,000), whereas molecular weight of most of the other 30 S ribosomal proteins ranges between 10,000 and 30,000 (13, 24, 27). Thus, P1(S1) might not be a “true” ribosomal protein, although the presence of P1(S1) in assay mixtures has been shown to stimulate poly(U)-directed fMet-tRNA binding (42). P1(S1) might be better considered a ribosomal “factor” bound to 30 S ribosomal subunits.

In many experiments we have found that reconstituted particles have polypeptide-synthesizing activity higher than the reference 30 S subunits. As discussed under “Results” in connection with variability of the activity data given in Table II, reconstituted particles with activity higher than reference 30 S subunits may reflect the real situation. This is consistent with the previous observation that the isolated E. coli 30 S subunits...
are partially deficient in some ribosomal proteins ("fractional proteins") (24, 41). Stimulation of the activity of isolated 30 S subunits upon addition of extra ribosomal proteins (24, 43) is also in agreement with the above interpretation. In this connection, it may be interesting to study the stoichiometry of each of the protein components in the isolated reconstituted 30 S particles obtained under the optimum conditions which ensure the maximum reconstitution efficiency.

As mentioned above, the observed weak "residual activity" of the protein-deficient particles described in the single component omission experiments (Table IV) could be explained, in some cases (such as the omission of P57(S7)), by presence of the omitted protein in other preparations as contaminants. However, in several other cases, the residual activity is too high to be accounted for by contamination of the omitted protein in other protein preparations. Whenever the possibility of such contamination existed in these cases, the protein mixtures prepared by omitting the pertinent protein were analyzed. The omitted protein in the protein mixtures was either completely absent or too small in amounts to account for the activity. High residual activity observed after omission of some proteins may suggest that the omitted protein is not directly involved in the function of assembled 30 S subunits, but has a role in the assembly process. We have obtained data indicating that Protein P1a(S16) belongs to this category. Alternatively, the omitted protein may be important to maintain an "active center" in a proper configuration of the reconstituted particles may have the "active center" to this category. Alternatively, the omitted protein may be of assembled 30 S subunits, but has a role in the assembly process.

The present reconstitution system may be useful in analysis of the role of each of the molecular components in the assembly process, as well as in ribosome function (1, 6, 8, 36). In addition, the present system offers an analytical method for detecting a protein which is identical or functionally equivalent to a particular 30 S ribosomal protein. We have already used the present system successfully to show that most, if not all, of the E. coli proteins have functionally equivalent counterparts among proteins extracted from Bacillus steaembachus 30 S subunits (15).

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