Assembly Mapping of 30 S Ribosomal Proteins from Escherichia coli

FURTHER STUDIES*

WILLIAM A. HELD, BYRON BALLOU,‡ SHOJI MIZUSHIMA,§ AND MASAYASU NOMURA

From the Institute for Enzyme Research, Departments of Biochemistry and Genetics, University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

Further studies were performed on the sequence of addition of proteins to 16 S RNA during the in vitro reconstitution of 30 S ribosomal subunits from Escherichia coli. Direct binding of protein S17 to 16 S RNA was studied in detail, and the following results were obtained: (a) under reconstitution conditions, a maximum of approximately 1 mole of S17 is bound per mole of 16 S RNA, either alone, or in the presence of all other 30 S proteins; (b) S17 binds only to 16 S RNA and not to 23 S RNA; and (c) radioactive S17-16 S RNA complexes are directly converted (without dissociation) to 30 S subunits by the addition of excess unlabeled total 30 S proteins. From these results, we conclude that the binding of S17 to 16 S RNA is specific. We have also determined the positions of S15, S16, S17, and S12 in the assembly map and have clarified subsequent interactions depending on these proteins. A revised assembly map is presented which incorporates the additional information obtained from these experimental results.

Previous studies from this laboratory (1) have demonstrated that in vitro reconstitution of Escherichia coli 30 S subunits occurs in a sequential, cooperative fashion. It was found that under reconstitution conditions only a few proteins bind directly to 16 S RNA. Those originally found were S4, S7, S8, S13, S17 (studied as a mixture of S16 and S17), and S90.1 Certain other proteins became bound only after some of the above proteins were bound. The remaining proteins required the presence of proteins in both of the above groups in order to be incorporated into the final structure. In this manner, the sequence of addition of proteins to the 16 S RNA was studied, and from the data obtained, an "assembly map" was constructed showing cooperative effects among the various 30 S ribosomal proteins in the assembly reaction (see Fig. 6, inset).

However, our initial studies on the assembly mapping of 30 S ribosomal proteins were incomplete (1). First, in these experiments the binding of protein S15 was not investigated. Schaup et al. (3) subsequently found that protein S15 binds directly to 16 S RNA. This observation has been confirmed by us and preliminary reports have been made with respect to this protein. Second, we did not use pure S16 and S17 in construction of the map, but a mixture of these two proteins. Third, because of technical problems, the position of the protein S12 in the assembly map was not determined. Finally, although we have demonstrated direct incorporation into 30 S subunits of 16 S RNA-protein complexes obtained after the individual binding of S4 and S8 to 16 S RNA, such rigorous proof of specificity of binding was not done with respect to other initial binding proteins. Subsequently, Schaup et al. (3, 6), as well as Garrett et al. (7), studied individual binding of 30 S proteins to 16 S RNA and concluded that the binding of S17 to 16 S RNA observed by us was nonspecific. In addition, both groups failed to observe binding of S13.

In this paper, we present results of further experiments designed to clarify the above mentioned uncertainties. From the data obtained, we present a revised assembly map which incorporates information obtained from these experimental results.

MATERIALS AND METHODS

Buffers—Buffer A: 30 mM Tris·HCl, pH 7.4 (24), 20 mM MgCl2, 0.1 mM 2-mercaptoethanol; Buffer B: Buffer A containing 0.5 M potassium chloride. The standard nomenclature of 30 S proteins (2) is used in this paper. Where appropriate, the previous nomenclature used in our laboratory is indicated in parentheses (1). Equimolar mixtures of 21 purified 30 S proteins are designated ΣSi. In experiments in which individual proteins are omitted from this mixture, the designation ΣSi-Sx is used where Sx is the protein omitted. Other abbreviations used are: TP30, unfractionated 30 S proteins extracted from 30 S subunits; R1, reconstitution intermediate; R1*, activated reconstitution intermediate.
Preparation of Ribosomes and Ribosomal Proteins—E. coli strain Q18 ribosomal proteins were used in most experiments (for exception, see below). The methods used for preparation of ribosomal subunits, 16 S RNA, and purification of 30 S proteins have been described (8, 9).

Preparation of radioactive proteins—In most of the experiments described in this paper, purified 30 S protein labeled in vitro with [3H]- or [14C]formaldehyde by reductive alkylation (10) were used. Each purified 30 S protein (200 µg in a volume of 0.2 ml) was dialyzed against borate-KCl buffer, pH 9.0, reacted with [3H]- or [14C]formaldehyde (New England Nuclear, specific activity 100 Ci per mole and 50 Ci per mole, respectively), followed by reduction with sodium borohydride. Alkylated proteins were then dialyzed against Buffer B to remove unreacted low molecular weight components. The specific radioactivity of each protein preparation was estimated by assuming that no protein was lost during dialysis and by determining the amount of trichloroacetic acid-insoluble material (radioactive material) formed. Corrections were made for counting efficiencies of samples on filters relative to the same samples in solution counted in Aquasol (New England Nuclear). The following molecular weight values were used for the above calculation (11): S6, 16,000; S13, 15,700; S16, 12,500; S17, 10,400; S12, 8,800.

Using the calculated value for specific radioactivity of the various labeled protein preparations, the maximum binding observed (in the presence of all other 30 S proteins) varied between 0.6 and 1.1 moles per mole of 16 S RNA for 10 Ci per ml of 30 S RNA and about 10 Ass, eq of protein (since 1 Ass eq of protein corresponds to be 160,000 counts/min per 20 µg of the purest protein as 14C eq). Corrections were made for counting efficiencies of samples on filters relative to the same samples in solution counted in Aquasol (New England Nuclear). The following molecular weight values were used for the above calculation (11): S6, 16,000; S13, 15,700; S16, 12,500; S17, 10,400; S12, 8,800.

Radioactive alkylation proteins were designated in this paper as [3H]- or [14C]CH3 proteins to distinguish them from radioactive proteins prepared by in vivo methods (see below).

Several investigators have used reductive alkylation to label proteins and have found that the procedure causes minimal changes in the structure of most proteins (10, 12). For example, Pon et al. (13) have reported that radioactive initiation factor IF-3 prepared in this manner is functionally active. We have also found that significant fractions of both [3H]CH3-S12 and [14C]CH3-S17 prepared by the present method are functionally active as tested by ability to be reconstituted into functionally active 30 S particles; the particles containing alkylated S12 or alkylated S17 were as active as the ones containing all normal proteins in poly(U)-directed polynucleotide polymerase assays. Functional reactivation of [3H]CH3-S6 was not performed since omission of S6 does not result in reduced function of reassembled 30 S subunits to any significant extent (9, 14). It should be kept in mind, however, that the procedure does involve chemical modification of amino groups, changing their pK values by about 0.5 pH unit (10). Thus, it is possible that the procedure alters the binding properties of proteins.

In some experiments, radioactive ribosomal proteins were used which were prepared from cells grown either in the presence of 8°S sulfate as a sole source of sulphur or in a medium containing a mixture of H labelled amino acids. In the former case, E. coli strain NO49 (W3110 str') was precultured in 20 ml of the following medium: NaH2PO4, 0.03 m; KH2PO4, 0.02 m; NH4Cl, 0.0075 m; MgSO4, 2 × 10−4 m; CaCl2, 3 × 10−3 m; FeCl3, 1 × 10−4 m; glucose, 0.2%. Cells were collected in mid-log phase (about 2 × 108 per ml) and washed with the same medium without glucose but containing 5 × 10−5 m MgSO4. Cells were then resuspended in the same washing buffer at a cell density of 2 × 106 per ml. Glucose (0.2%) and 20 mCi of [3H]sulfate were added (total volume, 200 ml); the cells were then allowed to grow to a density of about 109 per ml. Cells were then washed three times with the same medium without glucose but containing 0.05 m nonradioactive sulfate.

30 S ribosomal proteins were isolated from labeled cells after mixing with a suitable amount of unlabeled carrier cells and subjected to column chromatography on CM-cellulose and/or phosphocellulose by techniques described elsewhere (4, 9), except that proteins were extracted from the 30 S ribosomes with 4 M urea-2 M LiCl (8). [3H]-Labeled proteins were prepared from cells grown in a similar glucose synthetic medium supplemented with a [3H]-amino acid mixture (New England Nuclear, NET 250 l-amino acid-[3H] mixture; 5 mCi/172 µg of amino acids in a total of 50 ml of medium). Nonradioactive carrier cells were mixed with radioactive cells before making cell extracts.

Binding experiments—Individual purified 30 S proteins were dialyzed against Buffer B (9), and 16 S RNA was dialyzed against Buffer B. Labeled 30 S proteins were added to 16 S RNA in a 1:1 stoichiometry (as determined by acid precipitation of radioactive material per A260 eq of protein). The mixture was dialyzed against Buffer B for 6-8 hours at 0°.

RESULTS

Binding of S17 to 16 S RNA—As mentioned above, our initial binding experiments were done with a mixture of S16 and S17. We have subsequently found that radioactive S17 ([3H]S17, as well as [3H]CH3-S17) binds to 16 S RNA in the absence of other proteins, whereas S16 does not (Fig. 1 and Table 1). Other workers reported previously that S17 binds to 16 S RNA but concluded that the binding is nonspecific (3, 6, 7). Contrary to this conclusion, we believe that the binding of S17 to 16 S RNA under our conditions is specific. The evidence for this conclusion is as follows.

1. Increasing amounts of [3H]CH3-S17 were added to a constant amount of 16 S RNA. The binding of S17 to 16 S RNA increased and reached a saturation value at a molar ratio of protein added to 16 S RNA of about 3:1 (Fig. 2). The maximum amount of S17 bound to 16 S RNA was about 1.2 moles per mole of 16 S RNA calculated from the specific activity of the proteins used. In view of possible errors in the determination of the specific activity, however, we consider that this value is in reasonable agreement with a stoichiometry of 1 mole of S17 per
mole of 16 S RNA. This conclusion is strengthened by our observation that the saturation value for the binding of S17 to 16 S RNA by itself is close to that obtained in the presence of all other 30 S proteins (Fig. 1, a and b, and Fig. 2). The same conclusion was obtained when we used [3H]S17 instead of [14C]CH3-S17 (data not shown).

2. Garrett et al. (7) concluded that the binding of S17 to 16 S RNA is nonspecific because S17 binds equally well to 16 S and 23 S RNA. We, therefore, examined this possibility. [3H]-CH3-S17 was incubated with an equimolar mixture of 16 S RNA and 23 S RNA under standard reconstitution conditions, and then the binding was examined by sedimentation analysis using sucrose gradients made with either reconstitution buffer or Buffer C. In the former buffer, 23 S RNA tends to aggregate, and therefore, the latter buffer was also used. In both cases, labeled 16 S RNA was added to locate the position of 16 S RNA in the gradients. As shown in Fig. 3, S17 bound exclusively to 16 S RNA. No binding to 23 S RNA was observed.

3. Finally, we have examined whether the observed 16 S RNA-S17 complex represents a genuine intermediate in the in vitro reassembly reaction. The experiment was similar to the ones reported in our earlier paper (1) with respect to S4 and S8 binding. [3H]S17 or [14C]CH3-S17 was bound to 16 S RNA under standard reconstitution conditions. The complex was then mixed with a 3- to 5-fold excess of nonradioactive unfractionated 30 S proteins (TP30), and incubation was continued to assemble 30 S subunits. The resulting reconstituted 30 S subunits were purified, and the specific activity was compared with that of the original 16 S RNA-S17 complex. If the 16 S RNA-S17 complex is not an actual intermediate but a nonspecific aggregate, we would expect a large decrease in the specific activity as a result of dissociation of the initial nonspecific aggregate, mixing with excess nonradioactive proteins, and subsequent reincorporation into the reconstituted 30 S subunit in the alternative "correct" site. Alternatively, the initial nonspecific aggregate may prevent conversion of 16 S RNA to 30 S subunits. We found that the initial 16 S RNA-S17 complex was converted to 30 S subunits, and no decrease in the specific activity was observed (Table II). Control experiments, in which excess nonradioactive TP30 was added to radioactive S17 before mixing with 16 S RNA, showed an expected large decrease in the specific activity of the reconstituted 30 S subunits. Thus, we conclude that the 16 S RNA-S17 complex can be converted to 30 S subunits directly without dissociation.

From the above experimental results, we conclude that S17 binds specifically to 16 S RNA in the absence of all other 30 S proteins.

As already mentioned, in order to obtain approximately 1

![Graph](http://www.jbc.org)

Table I

| Experiment No. | 14C-Protein | 14C-Protein added | Unlabeled proteins added | CPM bound per 30 S unit | Relative amount bound % |
|---------------|-------------|-------------------|-------------------------|-------------------------|-------------------------|
| 1             | S17         | 2.2               | None                    | 1930                    | 63                      |
| 2             | S16         | 2.0               | None                    | 58                      | 4                       |
| 3             | S16         | 2.0               | S4, S20, S17            | 1456                    | 89                      |
|               |             |                   | 2S1-S17                 | 1380                    | 85                      |

1 A slight but significant increase in the binding of radioactive S17 was noted after the addition of excess TP30 to the preformed 16 S RNA-S17 complex, as shown in Table II. This can be explained by assuming that a small fraction of the S17 bound to 16 S RNA alone may be bound weakly and not recovered by the sucrose gradient centrifugation technique. However, the binding may be sufficiently strong so that it is not completely diluted by the excess TP30 added but is "chased" into 30 S ribosomes.
Fig. 2 (left). The binding of S17 to 16 S RNA alone or in the presence of all 30 S proteins. The binding of $[^{14}C]CH_3$-S17 to 16 S RNA alone (closed symbols) or in the presence of all other 30 S proteins (O) was determined as a function of the amount of $[^{14}C]CH_3$-S17 added. Two separate experiments, using different 16 S RNA preparations, are shown for the binding of S17 to 16 S RNA alone. The binding of $[^{14}C]CH_3$-S17 to 16 S RNA in the presence of all 30 S proteins (O) was performed at the same time as the experiment indicated by the △. The specific radioactivity of $[^{14}C]CH_3$-S17 used in these experiments was 2240 cpm per A$_{260}$ eq. The plateau values for binding of $[^{14}C]CH_3$-S17 to 16 S RNA alone or in the presence of all 30 S proteins corresponds to 1.2 and 1.4 moles of S17 per mole of 16 S RNA, respectively.

Fig. 3 (right). Binding of $[^{14}C]CH_3$-S17 to 16 S RNA in the presence of 23 S RNA. The binding of $[^{14}C]CH_3$-S17 to 16 S RNA was performed as described under "Materials and Methods" except the reconstitution mixture contained both 16 S and 23 S RNA extracted from 70 S ribosomes (6.8 A$_{260}$ units). A small amount of $[^{32}P]16$ S RNA was added as marker. $[^{14}C]CH_3$-S17 was added in about a 3 x excess over 16 S RNA. After heating at 40° for 1 hour, the reconstitution mixture was applied to a 5 to 20% sucrose gradients in (a) Buffer C and (b) reconstitution buffer. Centrifugation was for 3 hours at 45,000 rpm (Beckman SW 50 rotor). Fractions were collected and analyzed for A$_{260}$ and $[^{14}C]$ radioactivity. $[^{3}H]$ counts were corrected for a small amount of "spill" from $[^{32}P]$ (7.4%). It should be noted that 23 S RNA appears to form large aggregates which sediment to the bottom of the gradient when analyzed in reconstitution buffer (Fig. 3b).

Table II

Direct incorporation of 16 S RNA-S17 complexes into 30 S particles

| Experiment No. | Specific activity of the 16 S RNA-S17 complex or 30 S particles |
|---------------|---------------------------------------------------------------|
|               | (a) No TP30 added | (b) TP30 added at t = 0 min | (c) TP30 added at t = 30 min |
| 1             | 1492              | 3530                        | 2603                        |
| 2             | 2038              | 907                         | 2003                        |

mole of S17 bound per mole of 16 S RNA, it was necessary to add approximately 3 moles of labeled S17 (Fig. 2). This may reflect the presence of some inactive S17 in the preparation, weak binding of S17 to 16 S RNA, or loss of S17 due to adsorption to glass during the experiments. The results shown in Fig. 2 indicate that at low molar ratios of S17 to 16 S RNA, the amount of S17 bound to 16 S RNA alone is less than that bound when other 30 S proteins are present. This could be due either to prevention from the loss of S17 by other 30 S proteins, or to cooperative stimulation of the binding of S17 by other 30 S proteins. Under similar conditions, S16 alone does not influence the binding of S17. S4 and S20 together appear to stimulate binding somewhat (Table I), but we do not know whether this level of stimulation is significant.

Binding of S16—As shown in Table I, S16 by itself does not bind to 16 S RNA nor does it bind in the presence of S17 (Table I and Fig. 1c). Addition of S4 or S20 gives a small amount of stimulation, but addition of both S4 and S20 results in almost complete binding of S16, i.e., approximately 1 mole of S16 bound per mole of 16 S RNA (Table I and Fig. 1d). In our earlier work, in which a mixture of S16 and S17 was used as "P9," stimulation of the binding of the mixture of S16 and S17 (P9) by S4 (P4a) and S20 (P14), but not by S8 (P4b), was observed (cf. Fig. 1 of Ref. 1). Present experiments account for the previous experimental results on the basis of stimulation of S16 binding by S4 and S20 superimposed on the independent binding of S17.

Binding of S5—Our earlier work showed that binding of S5 depends on the presence of S8 and a mixture of S16 and S17 (P9) (1). Table III shows an example of earlier experiments in which a mixture of S16 and S17 was used, and binding of [3H]S5 was examined. [3H]S5 was bound in the presence of S4, S8, S20, and S21. In the legend to Fig. 7 of the paper by Held and Nomura (23), it is stated that "S17, but not S16, binds directly to 16 S RNA... As a mixture, both S17 and S16 bind to 16 S RNA... This statement was made based on unpublished preliminary experiments. In some earlier experiments, we tested the binding of nonradioactive S16 and S17 by analyzing the bound proteins by the two-dimensional electrophoresis technique. With this technique, the spot of S16 is very close to the spot of S17 as well as the spot of the oxidized form of S17 (9). When we examined the binding of S16 in the presence of S17, we found two major spots and thought that they were S16 and S17. In view of the experimental results described in this paper, we now believe that the previous statement "As a mixture, both S17 and S16 bind to 16 S RNA" is incorrect.
Table III

Stimulation of binding of [3H]S5 to 16 S RNA by other proteins

About 3 A260 eq of [3H]S5 were mixed with 4 A260 eq of nonradioactive proteins and 2.6 A260 units of 16 S RNA. Accurate specific activity of [3H]S5 used was not determined, and therefore, the actual amount of S5 corresponding to 100% binding could not be calculated. The reconstitution conditions used here and in the experiments shown in Table VI differed slightly from the conditions described under "Materials and Methods." Phosphoric acid rather than Tris was used in the reconstitution buffer, and incubation was for 40 min at 40°C.

| Other proteins added                  | Relative amount of S5 bound | %  |
|--------------------------------------|----------------------------|----|
| S4, S8, S20                          | 5                          | 50 |
| S4, S8, S20, S13, S16, S17           | 5                          | 50 |
| S4, S8, S20, S13, S16, S17           | 5                          | 50 |
| S4, S8, S20, S13, S16, S17, S7, S9, S14, S10, S3 | 86                         | 30 |
| ZSi-S5, S8                           |                            | 1  |
| ZSi-S5, S16, S17                     |                            | 1  |
| ZSi-S5                               |                            | 100|

S16, and S17 to the extent of about 50% of the maximum binding obtainable in the presence of all other proteins. Omission of S8 or both S16 and S17 from the above protein mixture or even from the complete protein mixture abolished S5 binding almost completely (Table III). We have now tested whether S16, S17, or both are required for binding of S5. As shown in Table IV, [3H]CH3-S5 did not bind to 16 S RNA alone, nor in the presence of S4, S8, S13, S16, S17, and S20 (see also Fig. 4a). Addition of S16 but not S17 to the complex containing S4, S8, S13, and S20 stimulates S5 binding very slightly, and addition of both S16 and S17 produced significant binding of S5 (Table IV and Fig. 4b). It should be noted, however, that the extent of S5 binding in the presence of S4, S8, S13, S16, S17, and S20 was still only about 20 to 30% of the maximum binding that can be obtained when all 30 S proteins are present (Table IV and Fig. 4). Thus, there is some quantitative difference between the present and the earlier experiments. Also, the sucrose gradient pattern in the present experiment suggested that a considerable amount of bound S5 was lost during centrifugation as evidenced by trailing of radioactive material from the A260 peak to the top of the gradient (Fig. 4b). It is possible that the labeling of S5 by reductive alkylation weakens S5 binding. Addition of all of the 30 S proteins appears to be sufficient to stabilize the binding.

Several proteins tested (S7, S9, and S15) did not significantly improve S5 binding (Table IV). S12 appears to increase binding of S5 slightly and as shown later, S5 appears to assist S12 binding.

We also examined the effect on S5 binding of omitting single 30 S proteins, in the presence of all other proteins (Table IV, Experiment 2). Omission of S17 or S15 had no effect while omission of S16 or S16 and S17 reduced binding to 60 and 40% of the maximum level, respectively. Again, some quantitative difference in the results was observed between these and earlier experiments. This difference may be related to the use of different protein preparations and slightly different reconstitution conditions in the two experiments (cf. legend to Table III). It is evident, however, that the effect previously observed with a mixture of S16 and S17 is mainly due to S16 and that S17 is also important for S5 binding.

Experiment 2

| Unlabeled proteins added | S5 bound | Relative amount of S5 bound |
|--------------------------|-----------|----------------------------|
| None                     | 79        | 3                          |
| S4, S8, S13, S20         | 73        | 2                          |
| S4, S8, S13, S20, S16    | 231       | 7                          |
| S4, S8, S13, S20, S16, S17 | 840   | 27                         |
| S4, S8, S13, S20, S16, S17, S7 | 724 | 23                         |
| S4, S8, S13, S20, S16, S17, S7, S9 | 3081 | 100                        |

Experiments was the same as that described in the legend to Table IV. The specific radioactivity of the [3H]CH3-S5 used in these experiments was calculated to be 4900 cpm per A260 eq. Based on this value, the amount of S5 bound in the presence of all 30 S proteins corresponds to 0.6 mole per mole of 16 S RNA. In Experiment 2, the amount of S5 bound in the presence of proteins S4, S8, S13, S20, S16, and S17 was somewhat less than in Experiment 1. This is probably due to variability associated with the somewhat weak binding of S5 obtained in the presence of these proteins and the gradual loss of bound S5 during centrifugation (see text for further explanation).

Fig. 4. Sucrose gradient sedimentation analysis of the binding of S5 to 16 S RNA-protein complexes. [3H]CH3-S5 was bound to 16 S RNA under reconstitution conditions in the presence of: (a) S4, S8, S13, S17, and S20; (b) S4, S8, S13, S16, S17, and S20; (c) ZSi-S5. The specific radioactivity of the S5 used in these experiments was the same as that described in the legend to Table IV.

Experiment 1

| Unlabeled proteins added | S5 bound | Relative amount of S5 bound |
|--------------------------|-----------|----------------------------|
| None                     | 79        | 3                          |
| S4, S8, S13, S20         | 73        | 2                          |
| S4, S8, S13, S20, S16    | 231       | 7                          |
| S4, S8, S13, S20, S16, S17 | 840   | 27                         |
| S4, S8, S13, S20, S16, S17, S7 | 724 | 23                         |
| S4, S8, S13, S20, S16, S17, S7, S9 | 3081 | 100                        |
TABLE V

| Unlabeled protein added | cpms/100,000 unit | Relative amount of S6 bound | % |
|------------------------|-------------------|-----------------------------|---|
| S15, --, --            | 46                | 2                           | 2 |
| S15, S11, --           | 44                | 1                           | 1 |
| S15, --, S18           | 1505              | 48                          | 48|
| S15, S11, S18          | 1877              | 60                          | 60|
| --, S11, S18           | 79                | 3                           | 3 |
| Σ S15-S6               | 3138              | 100                         | 100|

with about 0.7 to 0.8 mole of S15 bound per mole of 16 S RNA (data not shown). In addition, preliminary experiments also showed that a major part of the 16 S RNA-S15 complex can be incorporated directly into 30 S subunits upon mixing with a large excess of Tp30, followed by subsequent incubation under the reconstitution conditions. Thus, the binding of S15 to 16 S RNA is specific.

Effects of S15 on the binding of other proteins were then examined, wherever presence of such effects was suspected. In several cases which were examined, we did not find any effect (e.g. no effect on S6 binding; see Table IV). We have found, however, a strong effect of S15 on the binding of S18 and S6.

In our earlier work, the binding of S6 could not be observed in the presence of all of the initial binding proteins known at that time (S4, S7, S8, S13, S17 (added together with S16), and S20). In addition to these initial binding proteins, the presence of both S9 and S18 was necessary to get relatively good binding of S6. Under such conditions, the dependence of binding of S6 on the presence of S18 was clearly demonstrated (1). In addition, the binding of S18 was also known to depend on the presence of S6 (1).

We have now found that S15 strongly stimulates the binding of both S6 and S18. As shown in Table V, in the presence of S15 and S18, [3H]CH3-labeled S6 bound to the 16 S RNA-protein complex, and the extent of the binding was about 50% of the maximum binding which could be obtained in the presence of all of both S6 and S18. As shown in Table V, in the presence of S18 was clearly demonstrated (1).

In the absence of S15, S15 binding to S6 was completely (Table VII, Experiment 2). S15 stimulation to the value close to the maximum binding (data not shown).

Omission of single proteins during reconstitution using all the purified proteins indicated that S17 has a major role in S12 binding (Table VII, Experiment 2). Omission of S16 or S5 had little or no effect, but omission of S16 together with S17 caused a further decrease in the residual binding of S12 observed when S17 alone was omitted. Thus, we conclude that the major proteins required for S12 binding are S4, S8, S16, and S17. S5 stimulates but is not absolutely required for S12 binding.

Our earlier work showed that S12 does not bind to 16 S RNA singly, nor in the presence of S4, S8, and S20 (1) (see also Fig. 5). We have now found that further addition of S16 and S17 to the above mixture caused considerable binding (about 50% of the maximum binding). S20 did not appear to be necessary, but omission of any other component (S4, S8, S16, or S17) abolished S12 binding almost completely (Table VII, Experiment 1) (see also Fig. 5). Addition of S5 or the above protein mixture (S4, S8, S16, S17, S20) resulted in additional stimulation of S12 binding (to about 70% of the maximum binding). S15 might have a slight stimulatory effect. However, the other initial binding proteins, S7 and S13, did not show any significant stimulatory effect.

Omission of single proteins during reconstitution using all the purified proteins indicated that S17 has a major role in S12 binding (Table VII, Experiment 2). Omission of S16 or S5 had little or no effect, but omission of S16 together with S17 caused a further decrease in the residual binding of S12 observed when S17 alone was omitted. Thus, we conclude that the major proteins required for S12 binding are S4, S8, S16, and S17. S5 stimulates but is not absolutely required for S12 binding.

DISCUSSION

The revised assembly map is shown in Fig. 6. (For comparison, the assembly map published in the original paper (1) is also shown.) We have already discussed the main uncertainties connected with our earlier work. The revised map retains all of the previous RNA-protein and protein-protein relationships (shown with arrows), but several additional interactions are incorporated based on the experimental results described in the present paper. In addition, the relationship of S2 to S3 was added. It was previously found that S2 does not bind to 16 S RNA-containing complexes until most of the 30 S proteins are added to incubation mixtures (1). Single component omission experiments showed that omission of S3 as well as many

6 W. A. Held, W. R. Gette, and M. Nomura, manuscript submitted for publication.
Fig. 5. Sucrose gradient analysis of the binding of S12 to 16 S RNA-protein complexes. [3H]CH$_3$-S12 was bound to 16 S RNA under reconstitution conditions in the presence of: (a) S4, S7, S8, S13, S16, and S20; (b) S4, S8, S16, and S17; (c) ZSi-S12. The specific radioactivity of the S12 used in these experiments was the same as that used in the experiments described in the legend to Table VII.

Table VII

| Binding of S12 to 16 S RNA-protein complexes |
|---------------------------------------------|
| [3H]CH$_3$-S12 was used in Experiment 1 and [14C]CH$_3$-S12 in Experiment 2. The specific radioactivity of the [3H]CH$_3$-S12 was 2420 cpm per A$_{260}$ eq, and the [14C]CH$_3$-S12, 1680 cpm per A$_{260}$ eq. Based on these values, the amount of S12 bound in the presence of all 30 S proteins was approximately 1.05 and 0.6 moles of S12 per mole of 16 S RNA, respectively, in the two experiments. The discrepancy between these two values may, at least partly, be due to inaccuracy in determination of specific activity of radioactive proteins. |

| Unlabeled proteins added | S12 bound | Relative amount of S12 bound |
|--------------------------|-----------|----------------------------|
|                          | cpm/10$^5$ unit | %                          |
| Experiment 1             |            |                            |
| S4, S8, S7, S13, S16, S17, S20 | 1167     | 48                        |
| —, S8, S7, S13, S16, S17, — | 155      | 6                         |
| S4, —, S7, S13, S16, S17, S20 | 136      | 6                         |
| S4, S8, S7, —, S16, S17, S20 | 1108     | 49                        |
| S4, S8, S7, S13, —, S16, S20 | 1303     | 57                        |
| S4, S8, S7, S13, —, S17, S20 | 185      | 8                         |
| S4, S8, S7, S13, S16, —, — | 210      | 9                         |
| S4, S8, S16, S17 | 1137 | 47                        |
| S4, S8, S10, S17, S20 | 1037 | 42                        |
| —, S8, S16, S17, S20 | 67 | 3                         |
| S4, S8, S16, S17, S20, S15 | 1191 | 49                        |
| S4, S8, S16, S17, S20, —, S5 | 1028 | 79                        |
| S4, S8, S16, S17, S20, S15, S5 | 2443 | 100                      |
| ZSi-S12                    |            |                            |
| Experiment 2              |            |                            |
| ZSi-S12, S16 | 866 | 88                        |
| ZSi-S12, S17 | 396 | 40                        |
| ZSi-S12, S16, S17 | 246 | 25                        |
| ZSi-S12, S5 | 586 | 100                       |
| ZSi-S12, S3 | 933 | 95                        |
| ZSi-S12 | 987 | 100                       |

other proteins decreased binding of S2 (1). Since the effect of omission of these proteins (other than S3) could, at least partly, be explained through their indirect effects via S3, we included on the map only the information that S3 helps S2 binding. Effects of some other proteins, however, are too large to be explained on this basis alone. Nevertheless to avoid complications, we have simply indicated the presence of these other possible interactions by extra arrows pointed to S2 (see the legend to Fig. 6).

plained on this basis alone. Nevertheless to avoid complications, we have simply indicated the presence of these other possible interactions by extra arrows pointed to S2 (see the legend to Fig. 6).

It should also be noted that the arrows connecting initial binding proteins to 16 S RNA do not have any relationship to the actual order of binding sites along the 16 S RNA molecule. Several workers have studied the location of sites on 16 S RNA which bind these initial binding proteins (17-19). For example, Zimmermann et al. (19) have shown that S4, S8, S13, S15, and S20 bind to the 5'-terminal 900 nucleotides of the RNA while S7 binds to the 3'-terminal one-third of the molecule.
The reason for the discrepancy between the experiments by Schaup et al. (3, 6), as well as Garrett et al. (7), studied individual binding of 30 S proteins to 16 S RNA. There are two major discrepancies on the initial RNA-binding proteins between our conclusion and that obtained by these workers. One is concerned with the binding of S17, and the other is concerned with the binding of S13.

Both Schaup et al. (3) and Garrett et al. (7) concluded that the binding of S17 to 16 S RNA observed in our earlier experiments is nonspecific. In the present paper, we have presented evidence that the S17 binding is specific, and the 16 S RNA-S17 complex can be converted to 30 S subunits without dissociation. The reason for the discrepancy between the experiments by Schaup et al. (6) and those described here regarding the amount of S17 bound to 16 S RNA is not clear. In their first paper, Schaup et al. (6) observed no saturation plateau when they added up to 4 moles of radioactive S17 per mole of 16 S RNA. At this input protein to RNA ratio, they observed 2 moles of S17 bound per mole of 16 S RNA. The authors suggested that the nonspecific binding of S17 may be an artifact induced by reduction of the disulfide groups in the protein, implying that they had measured the binding of the reduced form of S17. In the subsequent paper, however, they reported that the reduced protein (a mixture of S16 and S17) was unable to bind to 16 S RNA under reconstitution conditions, and stated that the oxidized form, not the reduced form of S17, bound nonspecifically in their original experiments (3). In our experiments, we have always used the reduced form of S17. However, regardless of the oxidation state of S17, Schaup et al. (6) did not observe a clear plateau in the binding of S17. In the data shown for binding of S17 by Schaup et al. (6), a Bio Gel P-150 column was used to separate unbound protein from 16 S RNA. Since we have found that S17 sometimes aggregates in the absence of urea, it seems possible that some of the binding observed by these workers represented either binding of aggregated S17 to 16 S RNA or clution of aggregated protein in the void volume.

It is more difficult to explain the discrepancy between our experiments and those reported by Garrett et al. (7) concerning the binding of S17 to 23 S RNA. We did not observe the binding of S17 to 23 S RNA reported by these workers. The binding of S17 is completely specific for 16 S RNA. It is possible that the difference between our experimental results and their results could be accounted for by differences in the methods used for preparation and storage of purified proteins and RNA. In this regard, it is worthwhile to note that our S17 preparation is pure and not contaminated in any appreciable amounts by other proteins, including S16 or S15, as judged by NH2-terminal amino acid sequences of these protein preparations, as well as by immunoochemical methods. (The two-dimensional electrophoresis technique (20) generally used to check purity of ribosomal protein preparations is not sensitive enough to determine contamination of S17 by S16 or S10 because these three proteins migrate to a similar region, and, in addition, S17 usually gives two spots, presumably oxidized and reduced forms (9).) Furthermore, all of the proteins used in our experiments are highly active in the reconstitution of functionally active 30 S ribosomal subunits (9).

The second discrepancy is related to S13. In our earlier work, we observed weak binding of S13 to 16 S RNA alone. Both Schaup et al. (3) and Garrett et al. (7) did not observe such binding and speculated that the binding of this protein reported by Mizushima and Nomura (1) was caused by contamination of S13 by S15. The position of elution of S13 in our phosphocellulose chromatography used as a first step of protein purification is entirely different from that of S15 (9). No cross-contamination was observed when the preparations were examined by two-dimensional polyacrylamide electrophoresis. In addition, both Zimmermann et al. (19) and Craven and his co-workers observed direct binding of S13 under the same conditions. Moreover, the latter workers found that S13 binds only to 16 S RNA and not to 23 S RNA.

It is also our experience, however, that the degree of binding of S13 to 16 S RNA varies considerably. As reported earlier (1), S13 binding becomes almost quantitative in the presence of S4, S8, and S20. It is possible that the binding of S13 to 16 S RNA is weak, and some slight conformational changes which take place during purification or storage of the protein or RNA are sufficient to abolish binding to 16 S RNA alone. Such inactivation may be reversed, however, by direct or indirect interaction with other proteins such as S4, S8, and S20, resulting in strong binding to 16 S RNA in the presence of these proteins. Although we have not done further studies on the specificity of the weak binding of S13 to 16 S RNA, we have retained our original information in the revised assembly map.

As already emphasized in previous papers (1, 21), the arrows connecting proteins to proteins in Fig. 6 represent only the interaction (direct or indirect) that we detected during the experiments that were conducted to construct the map. Many other interactions which stabilize ribosomal structure probably occur. In addition, it is conceivable that proteins other than the initial binding proteins also interact with 16 S RNA in the finished ribosome structure. It should also be emphasized that cooperative interaction between two proteins shown in the assembly map does not necessarily mean direct interaction of the two proteins. For example, protein A may help the binding of protein B by directly interacting with B resulting in stabilization of the complex; alternatively, protein A may interact with either 16 S RNA or other proteins and may create a correct binding site for B indirectly through some conformational alteration.

It is probable that the "sequence" described in the assembly map corresponds, at least approximately, to the temporal sequence of assembly. Other evidence for this assembly sequence comes from the early kinetic studies of the assembly of 30 S subunits which showed that there is a rate-limiting unimolecular reaction that requires a high activation energy (22). At lower temperatures, a subset of the 30 S ribosomal proteins (RI proteins) interacts with 16 S RNA, and intermediate particles (reconstitution intermediate, or RI particles) accumulate. RI particles, when heated, form activated RI particles (RI*) which are capable of binding the remaining 30 S proteins at low temperatures. The temperature-dependent conversion of RI to RI* appears to involve a conformational rearrangement of the RI structure (23). In Fig. 6, those proteins found to be required for this temperature-dependent conversion of RI to RI* as well as three proteins (S20, S15, and S8) not required for the conversion but found in isolated RI particles (23), are shown above the dotted line. Thus, the incorporation of other proteins (under the dotted line) into the reconstituted 30 S particles appears to depend on the prior binding of RI proteins and the conformational change discussed above. All of the RI proteins thus identified occupy an earlier part of the assembly map which was constructed using quite different methods. It is evident from the complexity of the map, however, as well as the way in which the map was constructed, that the precise temporal order during the in vitro assembly would be difficult to determine. In fact, it is probable that there are several differ-
ent routes for assembly of 30 S ribosomal subunits, and that

differences in free energy of activation among them may be

erather small. Some evidence supporting this conclusion has been
discussed previously (21, 23).

We originally suggested that the assembly map reflects topo-

gological relationships between ribosomal proteins in the organized

ribosome structure (1). Several investigators isolated ribosome

fragments by mild RNase digestion and found that those proteins

that cluster together in such subparticles are, in general, the

proteins which show inter-relationships in the assembly map. The

correlation found between the fragment studies and the

assembly map has been discussed in detail by these workers in

connection with possible three-dimensional ribosomal structures

(24–27). Other investigators have used bifunctional cross-

linking reagents to find "neighborhood relationships" among

ribosomal proteins in the ribosomes. So far, the following pro-

tein dimers, or trimers, have been identified: S7-S9 (28, 29),

S8-S9 (30, 31), S13-S19 (29), S18-S21 (30, 32, 33), S2-S3.

The correlation found between the fragment studies and the

assembly map is hoped that the present assembly map will be useful in design-

ing further experiments to study structure-function relationships

as well as the mechanism of assembly of the 30 S ribosomal sub-

units.

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