Identification of the Proteins Associated with Subparticles Produced by Mild Ribonuclease Digestion of 30S Ribosomal Particles from Escherichia coli*

(three-dimensional structure/insoluble RNase)

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ABSTRACT Digestion of 30S ribosomal subunits from E. coli by insoluble ribonuclease produces three subparticles. The ribosomal proteins associated with each of these particles were identified. Some of the proteins are associated with only one of the three subparticles. The protein compositions of the three particles can be arranged in an overlapping linear sequence of five groups. Furthermore, inspection of the previously determined assembly sequence of the 30S proteins indicates that proteins associated in the subparticles are interdependent in the assembly process.

Several investigators have recently described conditions for mild degradation of Escherichia coli ribosomes by nucleases (1–7). In general, these workers have shown that controlled nuclease digestion can produce large subparticles from both 50S and 30S ribosomes. One conclusion developed in these experiments is that the three-dimensional structure of the native ribosome influences the susceptibility of the RNA to enzymatic attack. If this conclusion is correct, then the products of limited digestion by nucleases should reflect gross features of the three-dimensional structure of the ribosome. Thus, an analysis of the protein compositions of the particles released by nuclease action might give some clues to the three-dimensional arrangement of the proteins. Brimacombe et al. (7) have recently published experiments designed with this underlying concept. They examined several isolated fragments of the 30S ribosome and found that they contained different proteins. However, they were not able to identify the proteins.

We have developed conditions to digest the 30S ribosome with ribonuclease, with the aim of determining the distribution of the 21 proteins of this subunit among its resultant subparticles. Brief exposure of the 30S ribosome suspension to insoluble ribonuclease A produces three major products, sedimenting at about 22 S, 15 S, and 7 S. We have analyzed each subparticle for its protein composition. The 22S particle contains 15–16 proteins, the 15S particle has 12–13 proteins, and the 7S particle has 6–8 proteins. Each of the 21 proteins appears to be a component of at least one of the three particles. Comparison of the distribution of the proteins with their sequence of assembly, as determined by Mirushima and Nomura (8), suggests that those proteins that are directly

interdependent in the assembly reaction are associated on the same subparticles.

MATERIALS AND METHODS

30S ribosomes were prepared (9) from E. coli MRE-600 (10); the glassware was treated with diethyl pyrocarbonate (K + K chemicals) to inactivate ribonuclease (11).

Enzite insoluble RNase (Miles-Seravac, Ltd.) was washed with 0.5 M NaCl and resuspended in 0.01 M Tris·HCl–0.05 M KCl–0.01 M Mg (acetate) (pH 8.0). 30S ribosomes were dialyzed overnight against the same buffer. After the 30S ribosome suspension was heated for 10 min at 30°C, it was digested with 20 μg of Enzite RNase per A260 unit for 15 min at 30°C with constant stirring. The digest was cooled to 4°C and centrifuged at 15,000 rpm (27,000 × g) for 20 min.

The RNase-treated suspension of 30S ribosomes was desalted by gel filtration on Sephadex G-25 equilibrated with 0.01 M Tris·HCl (pH 7.2). The desalted digest was heated for 10 min at 56°C, and 3–5 A260 units were applied directly to a 5–20% sucrose gradient in 0.01 M Tris·0.05 M KCl–1 mM Mg (acetate) (pH 8.0). Centrifugation was for 3 hr at 50,000 rpm in a Spinco SW 50.1 rotor. For large-scale preparations, about 200 A260 units in 1–2 ml were layered on a 5–20% sucrose gradient and centrifuged in a Spinco SW 25.2 rotor for 16 hr at 25,000 rpm.

Ribosomal proteins were extracted by two methods: The first was with 66% acetic acid, as described by Hardy et al. (12). The second method provided direct analysis of a sample on polyacrylamide gels. Fractions were dialyzed against 0.01 M Tris–0.01 M MgAc (pH 8.0) and lyophilized to dryness. The powder was dissolved in 100 μl of 3 M guanidine·HCl–0.01 M Tris–5 μl 2-mercaptoethanol (pH 8.0) and applied directly to polyacrylamide gels.

For iodination of protein extracts, Na125I (New England Nuclear) was mixed with a solution of 2 mM I–0.02 M KI, to give a specific activity of 106 cpm/μmol (13). The protein was reacted in 8 M urea–0.2 M Tris·HCl (pH 8.0) with 250 mol of iodine per 250,000 daltons of protein. Slightly greater incorporation can be achieved with larger excesses of iodine, but the modified proteins do not migrate properly during polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis of the proteins was performed at pH 4.5 as described by Hardy et al. (12), with the methylene bianacylamide modifications of Voynow and Kurland (14). The gels were stained with Coomassie Brilliant Blue R 250 (Colab Laboratories, Inc.) in 12.5% CH3COOH for 12 hr and destained in 10% CH3COOH (15). Protein

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identifications were made on the basis of the comparisons described by Wittman et al. (16).

For analysis of the proteins containing $^{131}$I, 14-cm polyacrylamide gels were used. The gels were fractionated by a Gilson model B 100 gel crusher. The fractions were dissolved in 10 ml of toluene containing 5 g of 2,5-diphenyloxazole and 90 ml of BBS-3 Biosolve (Beckman Instruments) per liter, and counted in a Beckman LS-250 scintillation counter.

RNA was isolated from ribosomal particles according to Monier et al. (17). The ribosomal RNA was fractionated by electrophoresis in 3.5% polyacrylamide gels, as described by Peacock and Dingman (18).

RESULTS

Production of subparticles by RNase

Conditions for ribonuclease digestion of 30S ribosomes were sought that would cause a limited extent of RNA cleavage. Ribonuclease covalently attached to cellulose was chosen for the digestion as its insolvability not only greatly restricts its reactivity, but allows it to be readily removed from the reaction mixture.

30S ribosomes treated with insoluble ribonuclease show no change in their sedimentation profile on sucrose gradients over a wide range of enzyme concentrations and incubation times. However, examination of the ribosomal RNA after phenol extraction either by centrifugation through a sucrose gradient (Fig. 1a) or by polyacrylamide gel electrophoresis indicates that significant cleavage of the RNA has taken place. The digestion products appear to consist of several large fragments and some smaller material, suggesting that cleavage was limited to several highly sensitive sites.

Digestion of 30S particles was successfully dissociated into several subparticles by rapid removal of Mg$^{++}$ and K$^+$, and subsequent mild heating. A suspension of digested 30S ribosomes was passed through Sephadex G-25 equilibrated with 0.01 M Tris-HCl (pH 7.2), heated at 55-57°C for 10 min, and centrifuged in a 5-20% sucrose gradient (Fig. 1c). Two new major species are observed, with estimated sedimentation constants of 22 S and 15 S (see Fig. 3). In addition, we find residual 30S particles, and some material of about 7 S. The sedimentation values assigned to these subparticles are intended to be a convenient system of nomenclature; they were computed on the basis of proportional distances traveled with respect to the residual 30S component.

Several variables have a pronounced effect on the production of these subparticles. Although E. coli MRE-600 is essentially free of ribonuclease activity (10), it is important that extreme precautions be taken to avoid ribonuclease contamination during the preparation of the ribosomes, as heating in the absence of stabilizing ions apparently renders the preparation extremely sensitive to nuclease attack.

In addition, the duration and the temperature of heating are quite important. The conditions we describe were optimal for a particular preparation of 30S ribosomes. We have found that regardless of the care we take to prepare ribosomes, every preparation is slightly different in its response to the heating procedure.

Identification of the proteins associated with the subparticles

The proteins associated with the three ribonucleoprotein fragments of the 30S ribosomes were identified by polyacrylamide gel electrophoresis. Most proteins could be unambiguously identified with those already characterized (19). However, minor bands are often present, and it is difficult to decide whether these represent true components of the particles. We used densitometric tracing of the stained gels to try to quantitate the differences between the proteins associated with the three particles. Fig. 2 reproduces the patterns obtained.

In these experiments, protein S8 did not resolve; however, other experiments, as well as the electrophoretic analysis described below, allow an unambiguous identification of this protein. For proteins S9 and S11, assignments are subjective. Although these two proteins migrate as a single band when both are present, S11 in pure form migrates slightly more slowly than S9. In addition, the staining of S9 is much more intense than that of S11. On this basis, we have tentatively concluded that S11 is a component of the 22S fragment and S9 is a component of the 15S subparticle.

Similarly the pair of proteins [S12, S13] and the three proteins [S15, S16, S17] did not resolve. We have not been able to differentiate between S12 and S13, although the peak heights in Fig. 2 indicate that either one or the other is present both in the 22S and the 15S particle. We believe that the set of proteins [S15, S16, S17] is probably present only in the 22S particle, but we cannot rule out the possibility that any one of these three proteins is missing from all subparticles. The important conclusions from Fig. 2 are that a
class of proteins is either missing or very minor in one or the other of the two large particles, and that all proteins are present in at least one of the three particles.

Stoichiometry of the proteins

As the densitometer analysis of the stained gels only gives an approximation of the relative quantities of each protein, we sought a more precise estimation. Proteins extracted from the various particles were iodinated with $^{131}I_2$ in 8 M urea. All of the 21 ribosomal proteins of the 30S particle contain tyrosine and histidine (19). These groups are completely and selectively iodinated in 8 M urea at pH 8.5 (13). Thus, the relative incorporation of $^{131}I$ into the proteins should be proportional to the number of tyrosine and histidine residues present per protein molecule, and to the number of copies of the protein per particle.

The efficacy of this method was tested by measurement of the number of proteins in the 30S ribosome. The extracted proteins were iodinated with $^{131}I_2$ in 8 M urea and were separated by polyacrylamide gel electrophoresis. We estimated the relative quantities of each protein present by dividing the total radioactivity present in each protein band by its histidine content (19) plus twice its tyrosine content (19). Iodination under these conditions produces predominantly diiodotyrosine and monoiodohistidine (13). The values so obtained were arbitrarily normalized to protein S4. The resultant figures agree very well with the direct measurements of Vojnov and Kurland (14); this finding suggests that the incorporation and recovery of $^{131}I$ is proportional to the relative quantities of the proteins present.

This method was applied to the proteins that compose the 22S and the 15S particles to determine whether proteins observed as faint bands on polyacrylamide gels should be considered as legitimate components of the particles. Thus, we were able to conclude, after three experiments with each particle, that most of the protein peaks seen in the densitometer tracings of Fig. 2 were major components. The exceptions to this generalization are proteins S3 and either S12 or S13 of the 22S particle. No significant recovery of $^{131}I$ was obtained from the region normally occupied by S3, and the band corresponding to [S12, S13] contained only enough $^{131}I$ to account for one protein. This conclusion also pertains to the 15S particle and, in addition, we suggest that protein S6 must be missing from this subparticle.

Proteins S5 and S6 migrate as a single band through gels crosslinked with 0.75% methylene-bisacrylamide. However, proteins from the 15S particle in this region contain enough incorporated $^{131}I$ to account for one of the two proteins. Similarly on gels crosslinked with 0.15% methylene-bisacrylamide, protein S6 migrates with protein S3; again this region contains sufficient radioactivity to account for only one of the two proteins. Therefore, we suggest that protein S6 is not a component of the 15S particle.

Finally, it should be noted that several of the proteins were not found associated with a given particle. In several experiments, protein S1 was totally absent from the 22S subparticle. Similarly, proteins S2 and S3 were occasionally recovered from the 15S particle in low yields. Thus, firm conclusions about these proteins cannot be made without further study. A possibility is that these proteins are occasionally stripped from their respective particles due to the heat treatment or some other condition used during the preparation. If proteins may be dissociated from ribosomes under our conditions, then these same proteins could randomly reassort with all of the resultant particles. Indeed, such reassocation may explain the presence of proteins S1, S4, and S5 in all three particles. Hopefully, the isolation of other subparticles may settle this point.

**DISCUSSION**

We have obtained limited digestion of the 30S ribosome by brief exposure of it to insoluble ribonuclease. Our evidence indicates that only a few highly sensitive points of cleavage are attacked by the enzyme under the conditions used. The ribosome remains intact, but it can be dissociated into three smaller ribonucleoprotein particles by a short treatment at 56°C, in the absence of either Mg$^{++}$ or K$.^+$. These three particles were isolated and the proteins contained in them were determined.

In most cases it was possible to identify the proteins unequivocally. Fig. 3 summarizes the protein compositions of the three particles. The proteins fall into five distinct categories: (a) proteins unique to the 22S particle, (b) proteins unique to the 15S particle, (c) proteins common to only the 22S and the 15S particles, (d) proteins common to only the 22S and 7S particles, and (e) proteins common to all three particles.

The significance of these five groups of proteins is unclear.

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**Fig. 3.** Summary of our identification of the proteins composing the three subparticles produced by RNase digestion of the 30S ribosomes. The order of proteins in each group, A–E, is arbitrary.

**Fig. 2.** Densitometer scans of polyacrylamide gels of the proteins extracted from untreated 30S ribosomes and from the purified subparticles produced by RNase. The gels were stained with Coomassie Brilliant Blue and scanned at a wavelength of 540 nm in a Gilford model 240 spectrophotometer equipped with an automatic gel-scanning device. The polyacrylamide gels were crosslinked with 0.15% (right) and 0.75% (left) methylene-bisacrylamide.
However, it is tempting to speculate that they represent actual clustering of proteins along the ribosomal RNA backbone. If this were the case, then the relative order of the groups from left to right would represent the relative order of the proteins from one end of the RNA to the other. Thus, it is possible that the proteins are strung along the RNA chain like a string of beads and that the five major clusters of proteins are organized along the ribosomal RNA chain in the order depicted in Fig. 3. In support of this idea, it should be remembered that in order to obtain these subparticles we found it necessary to remove all ions that stabilize nucleic acid structure and then to apply heat, further disrupting the secondary structure of the RNA. Thus, our subparticles probably are unbroken lengths of unfolded RNA associated with those proteins that interact with that RNA. If this is correct, then these subparticles can be viewed as overlapping fragments, and the sequence of proteins existing in the native 30S ribosome is reflected by the overlapping relationships. It is interesting to note that protein S21, which we have placed within one group at its end, does not form a complex in a total reconstitution of 30S ribosomes when RNA is used that has the 3' end missing (20). Furthermore, proteins S21, S18, and S11, which form a single group, undergo intermolecular crosslinkage induced by the reagent tetranitromethane (Shih, C. T. & Craven, G. R., manuscript in preparation). However, proof of these speculations must await the isolation of many more overlapping fragments of the 30S ribosome.

Mizushima and Nomura (8) have recently determined interdependent relationships that are involved in the sequence of assembly of the 30S ribosomal proteins. They suggest that the resultant map of assembly relationships may reflect the topology of the proteins in the final 30S subunit. If this idea is correct, one might expect that those proteins that cluster together in a particle produced by partial cleavage of ribosomal RNA would also show interrelationships in the assembly process. Therefore, we have compared the protein composition of the three subparticles and the assembly sequence (Fig. 4). We have redrawn the assembly sequence as determined and revised by Mizushima and Nomura (ref. 8, and as cited in Nashimoto et al., J. Mol. Biol., in press). The protein compositions of the two major particles are indicated by filled circles. We find that the proteins composing any one of the subparticles are not randomly distributed throughout the assembly map, and conclude that proteins that appear to cluster together on ribosomal RNA fragments also are interdependent in the assembly process. This finding supports the suggestion (8) that the assembly relationships reflect the three-dimensional organization of the proteins in the 30S ribosome and, in addition, supports the use of protein reagents (9, 21) as probes of the gross features of the topology of the ribosome.

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