Three-Dimensional Organization of the 30S Ribosomal Proteins from *Escherichia coli*, I. Preliminary Classification of the Proteins

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Communicated by R. H. Burris, August 24, 1970

Abstract. 30S ribosomal subunits from *Escherichia coli* were reacted with three protein reagents. After reaction, the ribosomal proteins were extracted and examined; of the 20 proteins known to compose the particle, nine did not react with any of the three reagents. We have tentatively classified these proteins as "internal," and the remaining eleven as "external." A strong correlation was found between these results and the sequence of assembly. Those proteins which enter the assembly process early are classed as internal, whereas those proteins found to participate later in the assembly sequence are classed as external.

The 30S ribosomal subunit, isolated from *Escherichia coli*, consists of approximately 20 chemically distinct proteins1–4, present in no more than a single copy per particle4–7. While some of the proteins are primarily involved in assembly, others participate in various steps of protein synthesis8.

One salient problem remaining is to understand the three-dimensional arrangement of these proteins. Unfortunately, the use of x-ray crystallography to probe the three-dimensional structure of the ribosome is not currently feasible.

Chemical techniques, however, do seem to hold considerable promise toward elucidating the arrangement of the proteins. Protein chemists have developed and extensively studied reagents that can derivatize proteins. Many of these reagents can differentiate between "exposed" groups and "buried" groups in proteins. With some of these reagents, we classify the ribosomal proteins as to their relative accessibility.

Materials and Methods. Preparation of ribosomes: *E. coli* MRE 600 (ref. 9) was grown in a 500 gallon fermentor with a medium and conditions similar to those described by Kjelgaard and Kurland.10 Ribosomes were prepared by repeated ammonium sulfate precipitation and washing.11 The 70S ribosome fraction was finally dialyzed against 0.01 M Tris, pH 8.0–0.01 M MgCl₂ buffer to remove residual ammonium ions, and quick-frozen or dialyzed for preparation of 50S and 30S particles.

70S ribosomes were dissociated by dialysis against TKM buffer (10 mM Tris–10 mM KCl–0.5 mM MgCl₂, pH 8.0). The subunits were separated by zonal centrifugation, with a B4 rotor in a Spinco model L4 centrifuge. Centrifugation was for 9 hr at 39,000 rpm, in a gradient of 10–30% sucrose with a 40% sucrose cushion. We have applied 25,000–35,000 A₂₆₀ units of ribosomes per run, to give 6000–8000 A₂₆₀ units of pure 30S particles.
The purity of the 30S ribosomal preparations and the products of the derivatization reactions was analyzed by ultracentrifugation in a sucrose density gradient.

**Extraction of ribosomal protein:** Ribosomal proteins were extracted by the techniques of Hardy et al. Ribosome suspensions were brought to 0.1 M MgCl₂ and added to two volumes of cold glacial acetic acid. After 30 min of stirring this solution in the cold, the precipitated RNA was removed by centrifugation, and the supernatant was dialyzed against 6 M urea-0.02 M phosphate (pH 5.8) containing 5 mM dithiothreitol (Nutritional Biochemicals Corp.). This solution was used directly for either polyacrylamide gel electrophoresis or phosphocellulose chromatography.

**Protein isolation:** Chromatographic separation on phosphocellulose of the 30S proteins was performed exactly as described by Hardy et al.

**Protein identification:** In most instances the proteins could be unambiguously identified with those previously characterized by gel electrophoresis and phosphocellulose chromatography. Some proteins were also isolated and characterized by amino acid and peptide analysis.

**Reaction with iodoacetate:** The 30S ribosomes were suspended in TKM buffer, adjusted to pH 8.2, and treated with a 5-fold excess of [¹⁴C]iodoacetate acetic acid (New England Nuclear) over potentially available SH functions; after 20 min at 37°C, the reaction was terminated with a 10-fold excess of β-mercaptoethanol.

**Reaction with 2-methoxy-5-nitrotropane:** 30S ribosomes were reacted with 2-methoxy-5-nitrotropane (MNT) under conditions such that complete reaction was achieved without altering their sedimentation velocity. The buffer used was 20 mM veronal-10 mM KCl-5 mM MgCl₂, pH 8.5. The reaction (with a 1000-fold molar excess of MNT over ribosomes) was complete after 4 hr at room temperature.

**Trypsin reaction:**Trypsin covalently attached to cellulose (Gallard-Schlesinger) was found to selectively digest a portion of the 30S ribosomal proteins. The reaction was performed in TKM buffer with 1.2 mg/ml of cellulose-trypsin. The reaction, monitored by gel electrophoresis, was complete after 16 hr at room temperature. Trypsin was removed by centrifugation (the supernatant contained no residual trypsin activity).

**Results. Reaction with iodoacetate:** Of the 20 proteins identified as components of the 30S particle, 12 have been shown to contain cysteine. These sulfhydryls appear to be completely reactive in 8 M urea. To examine the reactivities of these sulfhydryl groups as they exist in the 30S ribosome, the native particle was derivatized with [¹⁴C]iodoacetate at pH 8.0. The resultant particles were examined by sucrose gradient centrifugation for any significant alteration of the particle shape caused by the iodoacetate reaction. The particle maintained its 30S sedimentation velocity, consistent with the results of Traut and Haenni.

The proteins were extracted from ribosomes derivatized with [¹⁴C]iodoacetate and separated on phosphocellulose (Fig. 1). There are four major radioactive peaks; the several minor peaks were not analyzed because they contained considerably less radioactivity than that predicted for a single cysteine residue and in several cases did not coincide with a protein peak.

Two of the four major peaks (proteins 1 and 15a, ref. 12) were identified readily by their position on the phosphocellulose chromatogram and by polyacrylamide gel electrophoresis. The other two radioactive peaks contained more than one component and were, therefore, further purified by gel filtration on Sephadex G-100 with a urea buffer. In each case, the radioactivity was then associated with a single protein component. The other two proteins derivatized were thus identified as proteins 11 and 12a.
Therefore, of the 12 proteins containing potentially reactive sulfhydryl groups, only four are reactive when present in the 30S particle, proteins 1, 11, 12a, and 15a.

**Reaction of 2-methoxy-5-nitro trope.** 2-methoxy-5-nitro trope specifically derivatizes amino groups. Not only does MNT appear not to be involved in significant side reactions, but the product also displays a unique adsorption maximum at 420 nm. 30S ribosomes were treated with a 50-fold excess of MNT (over a calculated 200 lysine groups per 30S particle) for 4 hr in a veronal–Mg²⁺ buffer. The kinetics of production of the lysyl-tropone adduct were followed by measuring the increase in absorbance at 420 nm. The reaction is essentially complete after four hr of incubation at room temperature.¹³

The ribosome–tropone adduct was examined by centrifugation. By this relatively crude criterion, there does not seem to be any significant change in configuration. Spectrophotometric analysis of the RNA fraction remaining after extraction of the ribosomal proteins with 66% acetic acid indicated that none of the 420 nm absorbance was due to an MNT-RNA product. Polyacrylamide gel electrophoresis of the extracted proteins showed no major differences in mobility of any of the bands. The MNT-derivatized proteins were separated by phosphocellulose chromatography (Fig. 2). Protein content was analyzed by absorption at 225 nm; the lysyl-tropone content was monitored at 420 nm. Comparison of this elution diagram with those obtained from untreated proteins revealed that a number of proteins had altered chromatographic behavior. Equally significant was the finding of a substantial class of proteins which do not have an altered elution position and contain no lysyl-tropone (all these pro-
proteins contain many residues of lysine). Those protein peaks that are essentially unaltered in chromatographic position and which, by calculation from known extinction coefficients\(^3\) contain less than 0.5 mol of lysyl-tropone per mol of protein, are indicated by numbers in Fig. 2. By these criteria, there are 14 proteins that are probably not derivatized and 6 proteins that are substantially modified.

**Reaction with insoluble trypsin:** Modification of the 30S particle with iodoacetic acid and MNT suggested that there is a group of 30S proteins that can be classified as relatively exposed. However, if the ribosome has a porous structure, some of those proteins classified as exposed may actually occupy an interior position within the organelle. Thus, a reagent was sought which would be restricted sterically to surface interactions. The reagent selected was trypsin, covalently attached to an insoluble cellulose matrix.

Fig. 3 illustrates the polyacrylamide gel electrophoresis pattern of proteins extracted from 30S ribosomes treated with insoluble trypsin for 16 hr, compared with the standard protein pattern (on the left) from untreated particles. The mobility of many proteins was altered substantially, a number of bands were lost, and several new bands generated by the treatment with trypsin. Despite

**Fig. 2.** Phosphocellulose chromatography of protein extracted from 30S ribosomes reacted with MNT. Conditions of chromatography described in Fig. 1. Protein was measured at 230 nm and the lysyl-tropone adduct at 420 nm. The lysyl-tropone content is indicated by the cross-hatched area.

**Fig. 3.** Polyacrylamide gel electrophoresis of total extracted 30S protein, and of protein extracted from insoluble-trypsin digested particles.\(^{16}\)
considerable proteolytic alteration, the particles remained 30S as determined by centrifugation in a sucrose density gradient. Thus, trypsin treatment appears to be an effective probe of surface architecture.

Although the gel patterns allow the identification of several proteins altered in their mobility by exposure to trypsin, they do not establish completely which proteins are unaffected. Hence, this information was corroborated by separation of trypsin-treated proteins on phosphocellulose (Fig. 4). Comparison of this elution profile with the normal profile in Fig. 1 indicates that a number of peaks are missing. In addition, the baseline has been made more irregular, probably because of the presence of fragments of partially digested proteins. However, there are a number of prominent fractions that have been identified as proteins originally present in the native 30S particle. Although it is not certain that the major components remaining after proteolytic digestion are in fact unaltered, one can conclude that if these proteins have been partially degraded, the degradation has taken place in a way that does not influence their mobility on phosphocellulose or on polyacrylamide gel electrophoresis.

**Summary of results:** Table 1 records the data obtained. The most striking result is that there appears to be one class of proteins consistently available for modification and a second class completely unmodified by any of the three reagents used. In addition, there is a category of components that react with one or two of the reagents but not all three. We tentatively propose that it is possible to catalogue proteins based on their relative reactivities with these three reagents. A protein that cannot react with any of the three reagents is classified as "internal," whereas a protein modified by any one of the three derivatizing
agents is classified as "external" (Table 1). It is emphasized that these classifications are tentative and must be verified by further investigations using other reagents.

**Discussion.** The experiments described suggest that the proteins composing the 30S ribosomal subunit can be characterized by their differential reactivities to protein modifying reagents. Because a protein fails to react with a given reagent does not necessarily mean that that protein is inaccessibly buried within the structure of the ribosome. The classification of internal and external proteins proposed here rests on the assumption that these proteins are potentially reactive when removed from the ribosome. Unfortunately, merely because an isolated protein reacts when removed from the ribosome does not imply that it contains exposed and potentially reactive groups in the ribosome. Nevertheless, we have carried out several experiments to examine this point. First, experiments with trypsin and MNT indicate that the proteins of the unfolded 16S particle described by Gesteland\(^{15}\) are considerably more reactive than in the 30S form. In addition, the isolated proteins are completely susceptible to tryptic digestion. Finally, recent experiments to be published elsewhere (E. Ward and G. R. Cra- ven), have shown that, whereas only six proteins can react with iodine when in the 30S particle, all the proteins react when they are extracted from the ribosome and are placed in the buffer used for ribosome reconstitution. Ultimately, one can conclude that a given protein is located internally only after an exhaustive examination with many reagents of different properties and reactivities.

Increased confidence in the significance of our classification system is generated by a review of the reconstruction studies of Nomura et al.\(^{8}\) All of the proteins

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**Table 1. Relative reactivities of the 30S proteins.**

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<th>Protein number</th>
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<th>Derivatized by MNT</th>
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</table>

* (+), reactive; (−), unreactive; (· · ·), does not contain cysteine, hence, not able to react with iodoacetate.
identified by these authors as requisite for the assembly of a 30S particle are classified here as internal. Such a correlation strongly suggests that the differential reactivities of the proteins reflect their gross topological relationships.

An even more impressive correlation is derived from a detailed examination of the entire assembly process, as recently described by Mizushima and Nomura. Their investigation of the step-by-step assembly reaction revealed a definite interdependence for the attachment of individual proteins. The relationships evolved from their binding experiments and the assignments from the present study are summarized in the assembly map of Mizushima and Nomura (Fig. 5). The proteins designated in this paper as internal are enclosed by a solid oval, whereas the external proteins are enclosed by a dashed-line oval. For reference, the nomenclature of Nomura et al. is included.

![Diagram of 30S ribosomal subunit assembly](image)

**Fig. 5.** The assembly relationships of the 30S proteins. Proteins identified as internal in Table 1 are enclosed by a solid oval; external proteins are identified by an oval with a dashed line. The top numbers refer to the notation system published by Hardy et al. and the bottom numbers refer to the nomenclature of Mizushima and Nomura, from whom the assembly map details are taken also.
Fig. 5 displays a striking relationship between the assembly pattern and the reactivities of the proteins with the added derivatizing agents. It is apparent that those proteins that are assembled early in the process are relatively internal in the final structure, while those proteins added to the ribosome in the last stages of the assembly sequence are classified as external in the particle. Similar conclusions have been reached by Chang and Flaks. Thus, the simple interpretation, that the assembly sequence is governed by protein-protein interactions, appears to be correct.

We thank Mrs. Carolyn Connors and Mrs. Jacqueline Smith for technical assistance. In addition we also acknowledge the use of the Biochemistry Department pilot plant, directed by Dr. John Garver and supported by U.S. Public Health Service grant FR-00214. The work was supported by U.S. Public Health Service grant 5 P01 GM15422-04.

Abbreviations: MNT, 2-methoxy-5-nitrotropone; TKM, Tris-KCl-MgCl₂ buffer.