Proc. Nat. Acad. Sci. USA
Vol. 69, No. 5, pp. 1327–1331, May 1972

Spatial Arrangement of Ribosomal Proteins: Reaction of the *Escherichia coli* 30S Subunit with *bis*-Imidoesters

(gel electrophoresis/crosslinking reagent/*bis*-(methyl)suberimidate)

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Communicated by Daniel E. Koshland, Jr., March 13, 1972

ABSTRACT **The 30S ribosomal subunit of *E. coli* was treated with the bifunctional reagent *bis*-(methyl)suberimidate. Crosslinked ribosomal proteins were identified as bands with increased molecular weight after electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. The pattern of crosslinked products was altered when unfolded subunits were used. Free ribosomal protein was not crosslinked. Several of the crosslinked products were cleaved by ammonolysis to form the original monomeric protein constituents. The low yields of the reactions necessitated the use of radioactive proteins and autoradiographic procedures. The crosslinked proteins were tentatively identified by coelectrophoresis of the radioactive ammonolysis products with carrier 30S protein in sodium dodecyl sulphate, and coelectrophoresis at pH 4.5 in buffers containing urea.**

Full understanding of the mechanism of protein synthesis requires structural analysis of the relative locations of the different ribosomal proteins with respect to each other and to the ribosomal RNA. Both in ribosome assembly and in the functioning of the mature ribosome in protein synthesis there is evidently a high degree of cooperative interaction among different proteins. While the nature of some of these interactions has been inferred from genetic studies (1, 2), from studies of ribosome assembly (3), and from studies of ribosomes deficient in specific proteins (4), all these approaches are limited in that they cannot demonstrate directly the actual physical locations of the implicated proteins. We are attempting to map the position of ribosomal proteins by a direct chemical approach. In the work reported here, we have used the bifunctional reagent *bis*-(methyl)suberimidate to crosslink neighboring ribosomal proteins in the intact 30S particle. The major crosslinked products have been analyzed with respect to their constituent proteins. Five of the twenty 30S proteins were identified in crosslinked material.

METHODS

Reagents. The *bis*-(methyl)imido esters of malonic, succinic, adipic, suberic, and sebacic acids were prepared from the corresponding dinitriles by the method of McElvain and Schroeder (5), and were stored under reduced pressure as their dihydrochlorides at room temperature over P_2O_5.

Bacteria and Ribosomes. Escherichia coli strain MRE 600 was grown, and its ribosomal subunits were prepared by described methods (6, 7), except that the ribosomes were washed in a buffer containing 1 M NH_4Cl. Radioactive subunits were prepared from cells grown in the presence of ^{35}S in a minimal medium. All the 30S ribosomal proteins contain either cysteine or methionine (8). The specific activity of the freshly prepared ^{35}S-labeled ribosomes varied between 250,000 and 700,000 cpm/μg of ribosomal proteins.

Crosslinking. Intact 30S subunits were dialyzed against a buffer containing 50 mM KCl–1 mM magnesium acetate–50 mM triethanolamine·HCl, pH 8.0 (Buffer A) and adjusted to 3 mg/ml (ϵ 260, 1% = 150). Unfolded subunits were made by dialysis for 20 hr against buffer A without magnesium and brought to 3 mg/ml; they had an $s_{20,w}$ of 21, as measured in an analytical ultracentrifuge. Free ribosomal protein prepared with LiCl and urea (9) was dialyzed against buffer A with 6 M urea. 0.1 Final volume of a freshly made solution of 250 mM *bis*-(methyl)suberimidate dihydrochloride in 500 mM triethanolamine (free base) was added to the ribosomes or ribosomal protein, and the mixture was incubated at 37°. The time of incubation was 60 min for intact ribosomes and ribosomal protein, and 15 min for unfolded subunits. Reactions were terminated by pipetting aliquots of 40 μl into 100 μl of the sample buffer used for sodium dodecyl sulfate (SDS) gel electrophoresis. This procedure stops crosslinking by dissociating the ribosome into free protein and RNA; as is shown below, free protein is not crosslinked under these conditions. In some experiments the reaction was stopped with excess NH_4Cl; the results were the same. Therefore, reaction of the crosslinking agent with ribosomal proteins is not dependent upon prior dissociation of the ribosomes by, for example, SDS.

Electrophoresis. Crosslinked samples were electrophoresed in SDS gels that contained 10% acrylamide and 0.27% *N,N'*-methylene-*bis*(acrylamide). The products of ammonolysis were electrophoresed in one of three types of gel: One was an SDS gel containing 15% acrylamide and 0.6% *N,N'*-methylene-*bis*(acrylamide); the two others were gels run in β-alanine at pH 4.5 with 8 M urea. Both of the latter types of gel contained 7.5% acrylamide, with either 0.2 or 0.8% *N,N'*-methylene-bis(acrylamide). All gels were stained with Coomassie brilliant blue (R 250). Details of the electrophoretic procedures have been published elsewhere (8, 10).

Ammonolysis. Slices of gel that contained stained bands of radioactive crosslinked protein were tied into a piece of dialysis tubing and dialyzed for 24 hr against a solution containing

Abbreviation: SDS, sodium dodecyl sulfate.

15 volumes of concentrated NH_4OH (density 0.88)–1 volume of glacial acetic acid–1% SDS (11). This procedures causes ammonolysis of amidine groups in the crosslinked proteins, with the liberation of free monomeric ribosomal proteins. The treated slices were dialyzed against several changes of distilled water, then against a buffer appropriate for gel electrophoresis.

Autoradiography. Stained gels containing protein labeled with ^{35}S were sliced longitudinally, dried under reduced pressure onto filter paper, and placed in contact with Kodak No-Screen medical x-ray film for periods between 5 and 30 days (12).

RESULTS

Reaction conditions

Triethanolamine buffers and KCl were used instead of Tris–NH_4Cl in order to avoid the presence of substances with amino groups, which can react with imido esters. Subunits dissolved in this buffer sedimented as 30S particles in sucrose gradients (Fig. 1), and remained active in the poly(U)-directed synthesis of polyphenylalanine. Although the crosslinking reaction is more rapid at pH values above 8 (13), reactions were done at pH 8.0 to avoid dimer formation. Incubation with *bis*-(methyl)suberimidate did not change the sedimentation behavior of the 30S particles (Fig. 1*C*). Crosslinking under the conditions used caused a loss of activity of only 15%. The significance of this partial inactivation is not clear, since the yield of the crosslinking reaction is low.

A series of bifunctional imido esters differing in the number of methylene groups between the reactive groups was tested. Of these, the derivatives of succinic (C_4), adipic (C_6), and suberic (C_8) acids gave essentially the same pattern of crosslinked bands on SDS gel electrophoresis. The sebacic acid derivative (C_{10}) precipitated ribosomes and the malonic acid derivative (C_3) gave little crosslinking. The C_8 reagent *bis*-(methyl)suberimidate was used in all the experiments reported here.

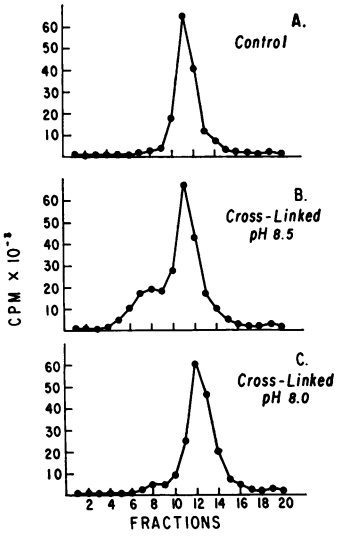

FIG. 1. Sucrose gradient analysis of crosslinked 30S ribosomal subunits. 30S subunits labeled with ^{35}S were dialyzed into the buffers described below and crosslinked with *bis*(methyl)-suberimidate. They were then dialyzed against 10 mM Tris·HCl (pH 7.4)–100 mM NH_4Cl–10 mM magnesium acetate; aliquots containing about 200,000 cpm were layered on 7–25% sucrose gradients in the same buffer. The gradients were centrifuged in a Spinco SW65 rotor at 60,000 rpm for 90 min, and 13-drop fractions were collected and counted. (*A*) Control subunits incubated in buffer A at pH 8.0 without *bis*(methyl)suberimidate. (*B*) As (*A*) incubated in buffer A at pH 8.5 with the addition of *bis*-(methyl)suberimidate. (*C*) Incubated in buffer A at pH 8.0 with *bis*(methyl)suberimidate.

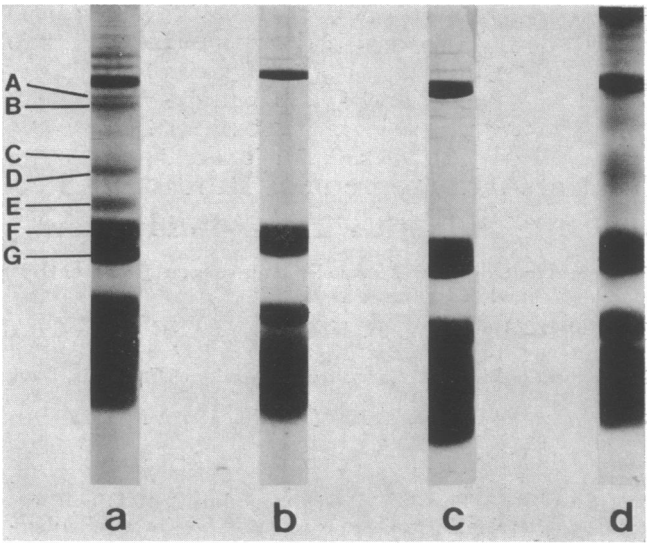

FIG. 2. Analysis of crosslinked ribosomal proteins in 10% acrylamide–SDS gels. (*a*) Crosslinked 30S subunits. The lettered bands are the crosslinked products referred to in the text. (*b*) Untreated control subunits. (*c*) Crosslinked free 30S ribosomal protein. (*d*) Crosslinked unfolded subunits.

Specificity of the crosslinking

Fig. 2*a* shows an SDS gel analysis of 30S proteins extracted from crosslinked subunits. Comparison with untreated 30S subunit proteins (Fig. 2*b*) shows several more slowly migrating bands. By contrast, when free ribosomal proteins were incubated with *bis*-(methyl)suberimidate at a concentration of protein equivalent to that in the experiment with intact subunits, no crosslinked bands were formed (Fig. 2*c*). Even when the free protein concentration was raised to 30 mg/ml, no reaction was evident. Subunits unfolded by dialysis against EDTA reacted with the crosslinking reagent, but in a different manner than the native particle (Fig. 2*d*).

The new bands apparent in Fig. 2*a* were not the only crosslinked products. The presence of other bands of crosslinked protein that coincided with normal protein bands was detected by the analysis described below. For example, the distance between bands *F* and *G* is increased after crosslinking; this suggested the presence of crosslinked material in this region. The major crosslinked products are lettered from top to bottom on the SDS gel shown in Fig. 2*a*.

Identification of the crosslinked proteins

The components of the crosslinked products were analyzed by cutting slices of the SDS acrylamide gel that contained crosslinked material and cleavage of the amidine crosslinks by ammonolysis. The ammonolyzed gel slice was then used as the sample in a second gel electrophoresis. New components of lower molecular weight were generated only from the

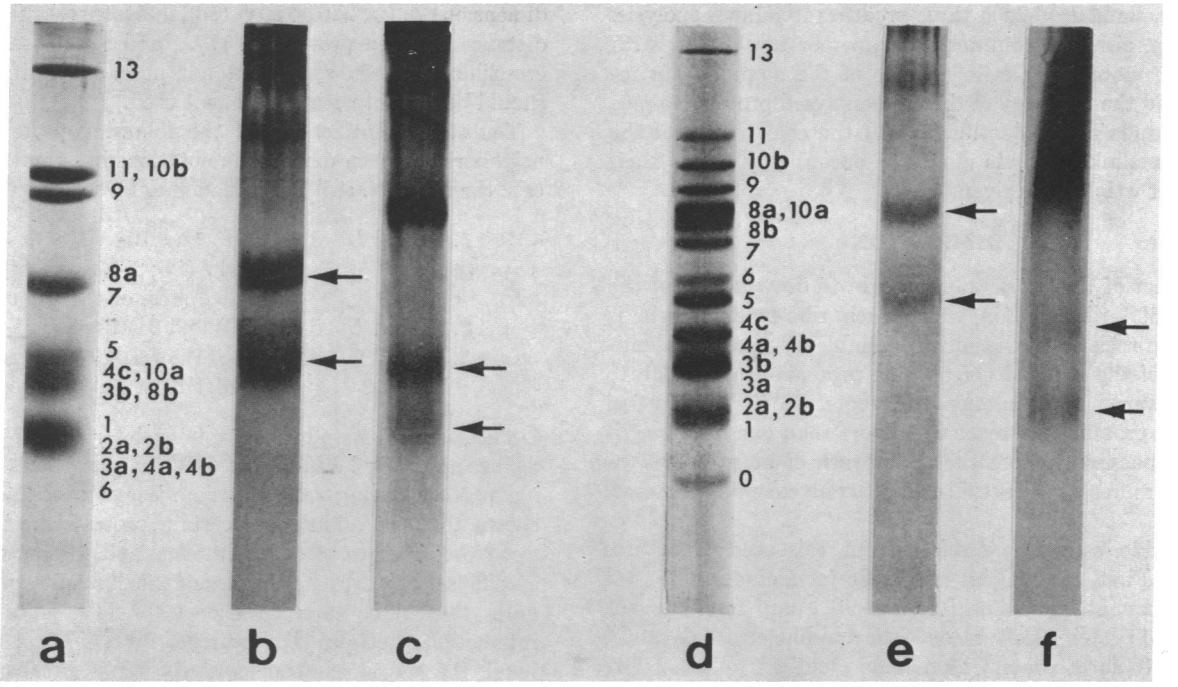

FIG. 3. Acrylamide gel analysis of the proteins contained in crosslinked bands *E* and *G*. *Fig.* 3*a–c*, 15% acrylamide–SDS gel analysis. (*a*) 30S ribosomal protein stained with Coomassie blue. (*b*) Autoradiogram of the ammonolysis products of band *E*. (*c*) Autoradiogram of the ammonolysis products of band *G*. *Fig. d–f*, analysis of 7.5% acrylamide gels run at pH 4.5 in the presence of 8 M urea. (*d*) Strained pattern of 30S ribosomal proteins. (*e*) Autoradiogram of the ammonolysis products of band *E*. (*f*) Autoradiogram of the ammonolysis products of band *G*. *Arrows* indicate the position of the released ribosomal proteins.

slices that contained crosslinked material. Analysis of protein in control slices taken from gels of uncrosslinked protein and subjected to the same conditions of ammonolysis showed no change in electrophoretic behavior. By this means, crosslinked proteins were found in bands undetected by visual inspection of the gel pattern due to coincidence with control bands. Because the reaction proceeds in low yield, ribosomes labeled *in vivo* with ^{35}S were used. Between 200,000 and 500,000 cpm were recovered in each slice containing protein to be analyzed, and about 20–30% of this material was successfully ammonolyzed. Each gel slice was mixed with (carrier) total 30S protein, ammonolyzed, and subjected to a second gel electrophoresis. Variable amounts of radioactive protein were trapped at the origin, and less than 10% of the crosslinked protein was recovered in bands of lower molecular weight. These amounts permitted their localization by autoradiography, with an exposure of 5–30 days. The radioactive bands on autoradiograms were compared with the stained pattern of carrier 30S proteins.

TABLE 1. *Characterization of the ammonolysis products*

Band	Apparent molecular weight	Molecular weight of ammonolysis products	Identification of proteins
A	57,000	30,000; 20,000; 14,000	—
B	53,000	30,000; 16,000; 11,000	—
C	44,000	30,000; 14,000; 11,000	—
D	37,000	20,000; 16,000; 13,000	7, 5, 10a
E	32,000	20,000; 16,000	8a, 5
F	28,000	14,000; 11,000	—
G	26,000	14,000; 11,000	4c, 2a

The numbered bands are identified in Fig. 2*a*. Their apparent molecular weight was determined from their position on 10% SDS gels, by use of the known molecular weights of the 30S ribosomal proteins for calibration. The molecular weights of the ammonolysis products (labeled with ^{35}S) were determined by electrophoresis on 15% SDS gels with added carrier 30S ribosomal protein. The position of the carrier proteins was revealed by staining with Coomassie blue, and that of the ammonolysis products by autoradiography. The identification of the products was made by urea gel electrophoresis, with a similar combination of staining and autoradiography.

Fig. 3 shows the identification of the components of crosslinked bands *E* and *G* (Fig. 2*a*) by SDS- and urea-gel electrophoresis of the ammonolysis products. Most of the material remains unreacted in the position of the original crosslinked band but, in addition, bands migrating more rapidly than the original band were formed. These were identified by comparison of the ^{35}S bands with the stained carrier 30S protein; the identification of the individual proteins found in each band in the total pattern has been described (14). Actual superimposition of an autoradiogram on a dried gel slice gave greater definition than is evident in the photographic reproduction. In addition, a third electrophoretic system, pH 4.5 urea gels with 0.2% bisacrylamide, which resolves bands 2*a* and 2*b* (6), was used.

The results of this analysis of the major crosslinked components are summarized in Table I. The molecular weight of each crosslinked band, and of the ammonolysis products formed from it, is given. The electrophoretic behavior of the products formed from bands *D*, *E*, and *G* in pH 4.5 gels in urea enabled their unambiguous identification as indicated in

the table. Band *D* yielded three products from ammonolysis. Therefore, either it contained a trimer or two dimers with the same mobility. The low yields of the ammonolysis reaction and the tendency of the ammonolyzed proteins to give diffuse bands made identification of the components of the other crosslinked bands difficult; accordingly, only their molecular weights are given.

DISCUSSION

The object of this work was to begin to draw a map of the *E. coli* 30S ribosome in which each ribosomal protein is located in relation to the entire ensemble by means of identification of the pairs of neighboring proteins near enough to be crosslinked by bifunctional reagents. On the assumption that one protein may react with more than one neighbor, it may be possible to construct a network of interconnections that will represent the actual spatial arrangement of ribosomal proteins.

Bis-imido esters were selected for this study for three reasons: First, the reagent is specific for amino groups, the most frequent amino acid functional group found among ribosomal proteins (15); second, the amidine crosslink can be cleaved to form protein monomers identical to the native protein constituents of the crosslinked dimer; and last, the product of reaction of an amino group and an imido ester retains the same charge as the unreacted protein. Thus, to a first approximation, the electrophoretic behavior of a protein is retained despite reactions with the free *bis*-imido ester or its partial hydrolysis product, and is changed only by those reactions that join two proteins.

Only a small part of the total protein was crosslinked under the conditions used. There was a low yield for the participation of specific proteins in dimer formation, and many proteins did not appear to be crosslinked at all. A low yield is interpretable in terms of a competition between different reaction pathways undergone by the *bis*-imido ester: The reagent can hydrolyze at both ends without reacting with an amino group; one end can react and the other hydrolyze; it can form an *intra*molecular crosslink; or it can form an *inter*molecular crosslink. Absence of crosslinking involving certain proteins may reflect either the fact that they are inaccessible to the reagent and do not react at all, or that lysine residues on neighboring proteins are not favorably situated, possibly because the overall distances between the proteins is too great. The fact that most of the crosslinked proteins fall into the group found by Craven and Flaks and their coworkers to be chemically or enzymatically modified when intact ribosomes were treated with trypsin (16, 17), iodoacetate (16), or 2-methoxy-5-nitrotropone (16) is consistent with the former explanation.

The extended length of the amidine crosslinkage is approximately 11 Å. If the reactive lysine side chains are assumed to extend up to 5 Å from the crosslinked protein backbone, then crosslinking could occur between two proteins within 21 Å of each other. Therefore, the fact that certain proteins can be crosslinked indicates that they are 21 Å or less apart. The occurrence of crosslinking is consistent with descriptions of the ribosome put forward by Spirin and Cox that indicate a large amount of intraribosomal (18) and protein–protein (19) interactions. A simplified model of the 30S ribosome that distributes 20 proteins of molecular weight 17,000 evenly throughout the volume of an oblate spheroid of dimensions 220 × 220 × 55 Å (20), indicates that the average distance between proteins is 17 Å, and again predicts that crosslinks between some, but not necessarily all, proteins should be formed with the reagent used.

Tentative identification of the following protein–protein neighbors has been made: The notations in parenthesis indicate the Madison and Berlin nomenclatures (21).

1. Band *D* 5 (P8; S9) and 10a (P3b + 3c; S6) and 7 (P5; S7). Any 2 of the 3 possible dimers or the trimer.
2. Band *E* 5 (P8; S9) and 8a (P4; S5)
3. Band *G* 4*c* (P7; S11) or (P10; S12) and 2a (P13; S19)

The assembly map of 30S proteins determined by Nomura defines an ordered addition of different proteins to the growing particle during reconstitution. These experiments have shown that the addition of certain proteins are dependent upon the presence of other proteins, and the map defines a specific set of interdependencies of this kind. It strongly suggests that these interdependencies reflect protein–protein interactions between the proteins involved. On the other hand, the map does not necessarily imply direct interaction between two proteins in the completed particle, since cooperative interactions and induced conformational changes, both of protein and of RNA, could also account for an assembly map of the type described. Clearly direct chemical studies on the location of proteins are required to confirm the interactions suggested by the assembly map.

When the results presented here are compared with the assembly map, no clear conclusion can be drawn. The incidence of crosslinking is not sufficiently extensive to permit a decision as to whether or not proximity or interdependence on the assembly map infers topological proximity in the ribosome. Protein 5 (P8, S9) is related to 7 (P5, S7) in the assembly map, and the two may constitute one of the crosslinked products in Band *D*. On the other hand, proteins 5 (P8, S9) and 8a (P4, S5), which are unambiguously crosslinked, are not closely related on the assembly map; this result suggests that they are brought together by a conformational rearrangement after their primary binding to the particle. Similarly, protein 2a (P13, S19) is not closely related to either of the possible crosslinked partners 4c (P7, S11) or (P10, S12); however, the exact position of P10 (S12) was not determined. The results reported here, therefore, suggest that protein–protein interactions other than those indicated by the assembly map occur. It is clear that crosslinking methods can contribute to the mapping of ribosomal proteins. Future studies of the type reported here will use bifunctional reagents of greater length and different chemical specificity in an attempt to enlarge the number of different crosslinked proteins.

NOTE ADDED IN PROOF

Two recent articles have described the isolation and characterization of ribonucleoprotein fragments derived from the *E. coli* 30S ribosome by digestion with ribonuclease [Brimacombe, R., Morgan, J. M. & Cox, R. A. (1971) *Eur. J. Biochem.* **23,** 52–60; Schendel, P., Maeba, P. & Craven, G. R. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 544–548]. The fragments were found to contain different, although overlapping, subsets of ribosomal proteins; the proteins of each fragment

were interrelated on the assembly map (4). Of the sets of crosslinked proteins identified here band *E* could be derived from just one of the fragments, while band *G* contains proteins from two different fragments. The latter result suggests that, although certain groups of proteins may interact sufficiently strongly to be isolable as a ribonucleoprotein unit, additional protein–protein interactions also exist.

This work was supported by grants from the U.S. Public Health Service (GM 1792401 to R.T.), Damon Runyon Memorial Fund (DRG-1127; to J. H.), General Research Support, and Cancer Research Funds from the University of California (to R.T. and J.H.), and a Grant-in-Aid from the American Heart Association (69-461 to R.T.). R. T. is an Established Investigator of the American Heart Association. The contribution of Mr. J. Wedgwood to early phases of the work is gratefully acknowledged. A preliminary report of the work has appeared: *Fed. Proc.* **30**, 1203 Abstr. (1971).

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