Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 3946–3950, October 1974

Diagonal Polyacrylamide–Dodecyl Sulfate Gel Electrophoresis for the Identification of Ribosomal Proteins Crosslinked with Methyl-4-Mercaptobutyrimidate

(protein–protein interaction/ribosome topography)

ANDREAS SOMMER AND ROBERT R. TRAUT

Department of Biological Chemistry, School of Medicine, University of California, Davis, Calif. 95616

Communicated by Edwin G. Krebs, July 1, 1974

ABSTRACT **A diagonal polyacrylamide–dodecyl sulfate gel electrophoresis procedure is described. Its utility is related to the use of the reagent methyl-4-mercaptobutyrimidate as a protein–protein crosslinking reagent. Crosslinking with this reagent occurs through the formation of intermolecular disulfide bonds. Oxidized proteins are separated in one dimension by electrophoresis under non-reducing conditions and in the second dimension under reducing conditions. All proteins except those derived from crosslinked species fall on a diagonal. Methods are described for the identification of the separated monomeric components of crosslinked species. The technique has been applied to the 30S ribosomal subunit of *Escherichia coli*, and the new crosslinked dimer, S4–S13, has been identified.**

We have previously described the synthesis of methyl-4-mercaptobutyrimidate and its utility as a bifunctional reagent capable upon oxidation of forming disulfide crosslinks between neighboring proteins in the 30S ribosomal subunit of *Escherichia coli* (1, 2). In these experiments (2), radioactive crosslinked products were isolated as components of increased molecular weight from one-dimensional polyacrylamide–dodecyl sulfate gels. The monomeric components of these crosslinked products were identified both by determination of the molecular weights of the individual proteins formed upon reduction of the crosslinked dimers and by immunochemical analysis of the oxidized crosslinked material. These methods had limitations. Unambiguous identification of the crosslinked proteins depended upon the fact that they possessed unique molecular weights, or that antibodies against each monomeric component were available. Therefore, we sought a more rapid and simple method for detecting and identifying crosslinked products formed with mercaptoimidate.

The fact that the protein–protein crosslinks consisted of disulfide bonds suggested a diagonal technique for their analysis. Ribosomal subunits are treated with methyl-4-mercaptobutyrimidate and then oxidized to form disulfide linkages. The proteins are extracted and separated by electrophoresis on polyacrylamide–dodecyl sulfate disc gels. The gels are then reduced and embedded in a polyacrylamide dodecyl sulfate slab gel and electrophoresis is performed again. The only components that fall off the diagonal are those that were in disulfide linkages prior to the reduction step. The sum of the molecular weights of the reduced products from any crosslinked species is equal to that of the original oxidized product. The reduced products from diagonal gels can be identified on two-dimensional gels containing urea (3, 4). We report here the methods for diagonal gel electrophoresis of 30S ribosomal proteins of *E. coli* and for removal of dodecyl sulfate from proteins eluted from specific spots on the diagonal gel slab and subsequent electrophoresis of the proteins in gels without detergent. It is shown that nearly all 30S ribosomal proteins from *E. coli* can be crosslinked under the conditions described. The application of the method to identify the new protein pair S4–S13 is presented.

METHODS

Preparation of Ribosomal Subunits. Radioactive 30S ribosomes were isolated from *E. coli* strain MRE600 grown in the presence of [^{35}S]sulfate (2). The specific activity of the ribosomal protein was between 9.5×10^7 and 11×10^7 cpm/mg of protein.

Crosslinking of Ribosomal Proteins with Mercaptoimidate. Pure 30S subunits (1.5 mg) were suspended in 0.5 ml of a buffer containing 50 mM triethanolamine hydrochloride, pH 8.0, 1 mM $Mg(OAc)_2$, 50 mM KCl, 3% 2-mercaptoethanol (TEA-SH buffer), allowed to react with 10 mM mercaptobutyrimidate at 1° for 20 min, and then oxidized with hydrogen peroxide. These procedures have been described previously (2) and have been shown to result in the formation of disulfide bonds between SH groups on adjacent ribosomal proteins. No intermolecular disulfide bonds form when proteins previously extracted from the ribosomes are subjected to the same treatment (1).

Extraction of Crosslinked Ribosomal Proteins. The oxidized incubation mixture containing intact 30S subunits was mixed with 2 volumes of glacial acetic acid and the proteins were extracted according to Hardy *et al.* (5). After removal of RNA by centrifugation, the protein solution was dialyzed against 7.5% propionic acid and lyophilized.

Diagonal gel electrophoresis

Electrophoresis of the Oxidized Proteins. A portion of the lyophilized, oxidized ribosomal protein (350–450 μg) was resuspended in 50 μl of buffer containing 1 mM $MgCl_2$, 50 mM KCl, 50 mM triethanolamine·HCl, pH 8.0, 4% sodium dodecyl sulfate, and 10 mM iodoacetamide. The iodoacetamide was included in order to block free protein sulfhydryl groups which might otherwise participate in disulfide interchange reactions at pH 8.0. The solution was incubated at room temperature for 30 min and then heated at 65° for 10 min. The sample was applied to a discontinuous polyacrylamide–dodecyl sulfate gel system (6) containing 13.5%

acrylamide. Electrophoresis was performed at 2 mA per tube (12 cm × 0.3 cm) for 5 hr.

Reduction of Disulfide Linkages. The gel was removed from the electrophoresis tube and incubated in 50 ml of a buffer containing 24 mM Tris·HCl, pH 8.8, 190 mM glycine, 0.1% sodium dodecyl sulfate and 3% 2-mercaptoethanol. Reduction was complete after 10 min at 65°. The gel was then dialyzed for 30 min against 50 ml of a buffer similar to that above, except that the pH was 6.8 and 2-mercaptoethanol was omitted.

Electrophoresis of Reduced Proteins Separated in the Disc Gel. A gel slab of the same polyacrylamide concentration as the first disc gel (13.5%) was prepared (4) with glass plates 26.5 cm × 15.8 cm separated by 3-mm spacers. The dialyzed, reduced disc gel was embedded at the origin of the gel slab. Electrophoresis in the second dimension was carried out at 25 V for 90 min and then at 90 V for 24 hr. The slab was stained in 0.55% amido black in methanol-acetic acid-water (5:1:5) and destained by soaking in several changes of the same solution.

Identification of ribosomal proteins

Extraction of Proteins from the Diagonal Gel. Stained spots from the slab gel which fell off the diagonal were cut out and suspended in 500 μl of buffer containing 100 mM Tris-acetate, pH 7.8, 1% dodecyl sulfate and 1% 2-mercaptoethanol. The pieces of gel were pulverized and the suspension was heated at 65° for 10 min. Solid urea was added to a final concentration of 8 M and 300–400 μg of nonradioactive 30S ribosomal protein was mixed with the suspension. The amido black and dodecyl sulfate were removed by a modification of the procedure of Weber and Kuter (7). Samples were applied to a 1.0-ml column of Dowex AG1-X8 (20–50 mesh) (Bio-Rad) that had been previously equilibrated with buffer containing 50 mM Tris-acetate, pH 7.8, 8 M urea and 1% 2-mercaptoethanol. The protein was eluted with 500 μl of 66% acetic acid. The colorless protein sample was dialyzed against 7.5% propionic acid for 15 hr. Radioactivity of an aliquot of the dialyzed solution was measured and the rest was lyophilized. This procedure removed dodecyl sulfate quantitatively as shown by experiments with dodecyl [^{35}S]sulfate.

Two-Dimensional Electrophoresis of Proteins in Polyacrylamide Urea Gels. The ribosomal proteins were then separated by two-dimensional polyacrylamide gel electrophoresis (3, 4). Samples of radioactive protein were resuspended in 100 μl of a buffer similar to that used for electrophoresis in the first dimension but which also contained 8 M urea and 10 mM iodoacetamide. The sulfhydryl groups were alkylated to eliminate possible changes in the mobility at pH 8.7 due to their partial ionization (1). The gel slabs were stained with Coomassie blue, destained, dried onto a filter paper (4) and then exposed to Kodak No-Screen Medical x-ray film for 2–30 days, depending upon the amount of radioactivity applied to the gel. Between 9,000 and 30,000 cpm of proteins representing reduction products on the diagonal gels were analyzed.

MATERIALS

2-Mercaptoethanol was obtained from Schwarz/Mann; hydrogen peroxide AR 30% solution from Mallinckrodt Chemical Works; sodium dodecyl sulfate "Salzfrei" from Serva, Heidelberg; urea, Ultra pure, from Schwarz/Mann; iodoacetamide, from Calbiochem. Acrylamide and bisacrylamide were used without recrystallization as obtained from Eastman Organic Chemicals. Triethanolamine was also obtained from Eastman and was redistilled before use.

RESULTS

Diagonal Gel Electrophoresis of Protein from Unmodified 30S Ribosomes. Fig. 1a shows the diagonal gel pattern of 30S ribosomal proteins extracted from untreated subunits. Both dimensions of the separation were performed under reducing conditions. The protein of highest molecular weight, S1, is on the diagonal. That the diagonal line is not at 45° is due to the fact that the second dimension gel slab is deliberately made longer than the first dimension disc gel to increase resolution. All of the spots on the diagonal correspond to monomeric ribosomal proteins which are separated according to molecular weight. Both dimensions of the diagonal gel were calibrated with respect to molecular weight to obtain the scales indicated in Fig. 1c. The calibration was made by plotting the migration distances of standard proteins, monomeric ribosomal proteins, and well-established (see legend to Fig. 2) crosslinked dimers from the 30S subunit. The calibration curve for the first dimension of the electrophoretic analysis of representative single 30S proteins and dimers is shown in Fig. 2. The most important conclusion from this experiment is that the protein dimers fall on a straight line with monomeric proteins. This means that the apparent molecular weight of a crosslinked product is equal to the sum of the molecular weights of its monomeric components, as determined by this technique. That this is true makes it possible to draw conclusions concerning the identity of the components of crosslinked products solely on the basis of molecular weight data. Ambiguities in identification are due to the fact that many ribosomal proteins have nearly identical molecular weights.

Diagonal Gel Electrophoresis of Proteins Extracted from Oxidized SH-Charged (2) Ribosomes. Ribosomal subunits were allowed to react with methyl-4-mercaptobutyrimidate, oxidized with hydrogen peroxide, and analyzed by diagonal gel electrophoresis without reduction in both dimensions. The result is shown in Fig. 1b. As in the case of the control, all the protein spots fall on the diagonal line. However, as a result of the crosslinking procedure a number of products of increased molecular weight appear. These products are evident as a nearly solid stained continuum between S 1 and S 2 (the first and second spots descending from upper left in Fig. 1a) and as stained material to the left of and above the S1 band.

Separation of the Monomeric Components of Crosslinked Products. The crosslinking that takes place in the oxidation step, after reaction of ribosomes with methyl-4-mercaptobutyrimidate, results from the formation of disulfide bonds between sulfhydryl groups on appropriately neighboring proteins. The usefulness of methyl-4-mercaptobutyrimidate as a crosslinking reagent rests primarily in the fact that the components of dimers or oligomers formed with it can be regenerated by reduction of the disulfide linkages (1, 2). Sun *et al.* (2) showed that bands of increased molecular weight could be extracted from one-dimensional polyacrylamide dodecyl sulfate gels, and the molecular weights of their mono-

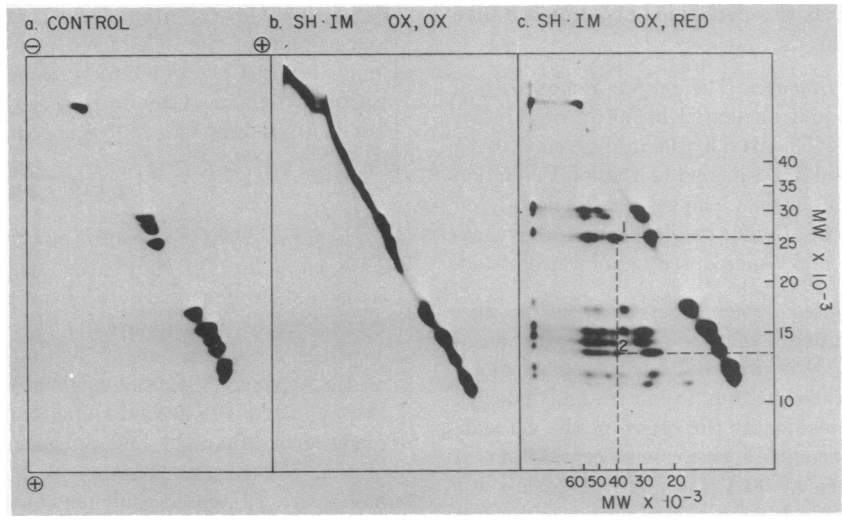

FIG. 1. Diagonal gel electrophoresis of 30S ribosomal proteins. (a) Protein extracted from unmodified 30S ribosomal subunits. Free sulfhydryl groups were blocked with 10 mM iodoacetamide before electrophoresis in the first dimension. (b) Protein extracted from 30S ribosomal subunits that had been modified with methyl-4-mercaptobutyrimidate (SH·IM) and oxidized with hydrogen peroxide. Free sulfhydryl groups were alkylated with iodoacetamide, and electrophoresis in both dimensions was carried out in the absence of reducing agents (OX·OX). (c) Protein extracted as in (b) from 30S ribosomal subunits labeled with ^{35}S. The electrophoresis in the first dimension was performed without reducing agent, while that in the second dimension was performed after reduction (OX, RED). The first dimensional disc gel was incubated in 50 ml of a buffer containing 24 mM Tris·HCl, pH 8.8; 190 mM glycine; 0.1% dodecyl sulfate and 3% 2-mercaptoethanol for 10 min at 65°, and dialyzed for 30 min against 50 ml of buffer similar to that described above except that the pH was 6.8 and 2-mercaptoethanol was omitted. Samples containing 350 μg of total protein were applied to each gel. Details of the electrophoretic procedure are given in *Methods*. Separation in the first dimension is from left to right; in the second dimension from top to bottom. The molecular weight (MW) scale for the first dimension (bottom, Fig. 1c) was established as described in the legend to Fig. 2. The scale for the second dimension was established with the values for ribosomal proteins determined by the procedure of Sun *et al.* (2).

meric components could be established by reduction of the extracted material followed by a second electrophoresis in dodecyl sulfate. The existence of a dimer consisting of S 2 and S 3 was established in this way.

The diagonal technique described here obviates the need to fractionate the first dimensional gel and to analyze separately each fraction. The first dimensional electrophoresis is performed under conditions in which disulfide linkages are retained. Then, prior to electrophoresis in the second dimension, the entire first dimensional disc gel is subjected to mild reducing conditions. The only proteins whose mobility is altered by reduction are those that were previously linked by disulfide bonds. These proteins therefore migrate more rapidly in the second than in the first dimension and as a result no longer fall on the diagonal. The resulting single gel slab shows all the reduced components, separated and identified by molecular weight, contained in all the crosslinked products in any given experiment.

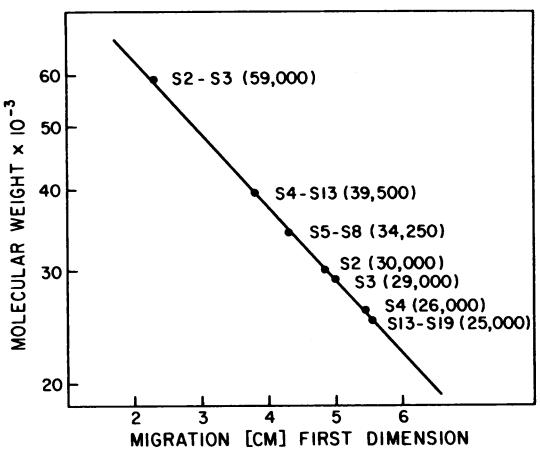

FIG. 2. Molecular weight calibration curve for the first dimension. The migration distances of ribosomal proteins and those crosslinked dimers established by other techniques [see Sun *et al.* (2) for review] and identified by techniques similar to those described here for S4–S13, were plotted against their molecular weights (also given in parentheses) on a semi-logarithmic scale.

A typical result with the 30S ribosomal subunit is shown in Fig. 1c. There are many protein spots that fall off the diagonal. Comparison of Fig. 1c with Fig. 1a and b shows that the removal of high-molecular-weight crosslinked material is complete. Fig. 1b shows that there is a continuum of convergent crosslinked products that are hardly resolved. However, the products of individual crosslinked species are apparent as only those reduced protein spots that fall on vertical lines. The intercepts of any ordinate line with the diagonal line or with the molecular weight scale indicate the position of the original crosslinked species and its molecular weight. The molecular weights of the reduced products are obtained from the calibration scale for the second dimension.

An example is illustrated by the dotted lines shown in Fig. 1c. The vertical line drawn through spots marked 1 and 2 indicates that the molecular weight of the original crosslinked species was 39,000. Only the two reduced spots that are numbered fall on this ordinate. The molecular weights of the reduced components are 26,000 and 13,500. The sum of the molecular weights of the reduced products is equal to that of the crosslinked material that gave rise to them upon reduction. It can be concluded that the proteins marked 1 and 2 had been joined as a disulfide linked dimer.

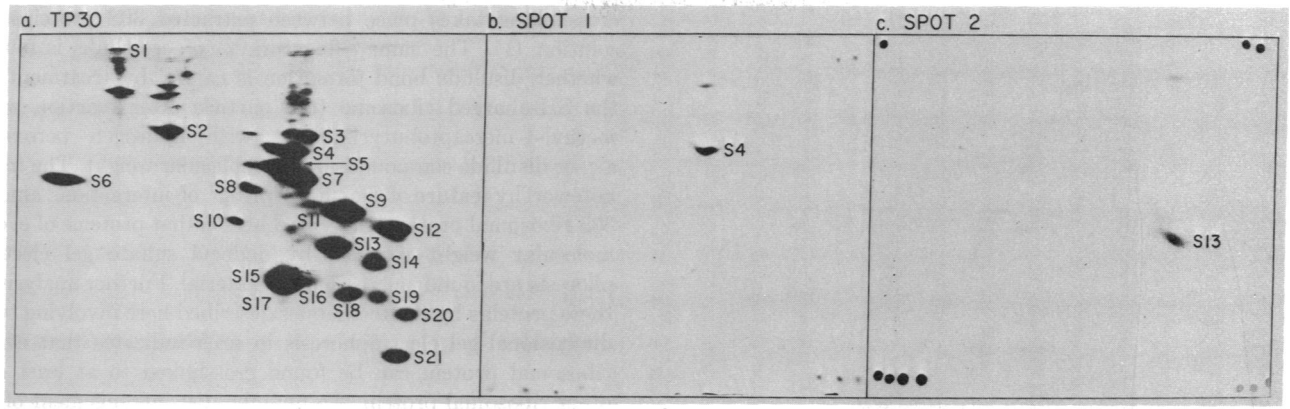

FIG. 3. Identification of components of crosslinked products by two-dimensional polyacrylamide–urea gel electrophoresis and radioautography. (a) Two-dimensional electrophoretic pattern of 30S proteins stained with Coomassie brilliant blue and numbered according to Wittmann *et al.* (12). (b) Identification of the component found in spot 1 indicated in Fig. 1c. The figure is a radioautogram of material extracted from spot 1 as described in *Methods* (30,000 cpm). The gel, which contained 350 μg of nonradioactive total 30S protein as carrier mixed with the radioactive material, was stained to give a pattern similar to that in Fig. 3a, dried, and radioautographed on Kodak No-Screen x-ray film for 17 days. The developed radioautogram was superimposed precisely on the stained gel (radioactive ink spots used for alignment are visible in the corners of the figure) and the position of the radioactive spot was determined. (c) Identification of the component found in spot 2 indicated in Fig. 1c. The procedure was identical to that described in (b), except that 22,000 cpm were extracted from spot 2, and exposure of the two-dimensional gel to x-ray film was for 22 days.

Identification of the Crosslinked Ribosomal Proteins. Electrophoresis in polyacrylamide–dodecyl sulfate gels does not resolve all ribosomal proteins, since many of them have nearly identical molecular weights. However, certain proteins, e.g., S1, S2, S3, and S4, do have unique molecular weights and have unique mobilities during electrophoresis in dodecyl sulfate. It is apparent that spot 1 in Fig. 1c corresponds to protein S4. The identity of the protein(s) present in spot 2 cannot be established unambiguously since there are two proteins, S13 and S14, in the large spot of molecular weight 13,500 daltons on the diagonal.

In order to establish unambiguously the identity of spot 2, a diagonal gel experiment was performed with 30S ribosomes labeled with ^{35}S (see *Methods*). Spot 2 was carefully cut out of the diagonal gel slab and the protein was eluted and mixed with total 30S ribosomal protein. Both stain and dodecyl sulfate were removed by passing the eluate through a column of Dowex AG1-X8, and the proteins were analyzed by two-dimensional gel electrophoresis in buffers containing urea (3, 4). The gel slab was stained to reveal the typical pattern shown in Fig. 3a. It was then dried and autoradiography was carried out to identify the position of the radioactive component(s) corresponding to spot 2. Fig. 3c indicates by superimposition of the autoradiogram on the stained gel from which it was made (not shown, but similar to Fig. 3a) that spot 2 contains a single protein, S13. A similar experiment was performed to confirm the identification of S4 in spot 1. That result is shown in Fig. 3b. The combined analysis of spots 1 and 2 by diagonal gel electrophoresis in dodecyl sulfate and by two dimensional gel electrophoresis in urea lead to the conclusion that the *E. coli* 30S proteins S4 and S13 were crosslinked with methyl-4-mercaptobutyrimidate.

DISCUSSION

The technique described here, diagonal polyacrylamide–dodecyl sulfate gel electrophoresis of proteins crosslinked by disulfide bonds, should be of general utility in the analysis of protein–protein interactions in complex systems containing multiple polypeptides. The technique is based upon and exploits a particular feature of the crosslinking reagent methyl-4-mercaptobutyrimidate: the crosslinks formed are readily cleavable under conditions that do not alter the molecular weight or the electrophoretic mobility between pH 4.5 and 8.7 of the cleaved products. The technique greatly simplifies the analysis of protein–protein interactions in systems containing many protein components.

The experimental system to which it was applied here, the 30S ribosome of *E. coli*, illustrates the usefulness of the technique. On the single gel slab shown in Fig. 2c can be found all the crosslinked dimers previously reported, S2–S3, S5–S8, S5–S9, S7–S9, S13–S19, and S18–S21. In addition, many previously unreported dimers can be identified (A. Sommer and R. Traut, manuscript in preparation). The dimer identified here, S4–S13, had not been reported previously and its identification adds to the rapidly accumulating information on the protein topography of the 30S ribosome.

The apparent proximity of proteins S4 and S13 as indicated by crosslinking is consistent with other evidence. K. Huang, R. Fairclough, and C. Cantor (personal communication, manuscript in preparation) have found efficient energy-transfer between fluorescent derivatives of these proteins in reconstituted 30S subunits. The proteins had been placed adjacent to each other in a model of the 30S ribosomes, constructed from a variety of experimental evidence from many laboratories, prior to the direct evidence reported here (8).

The diagonal gel system described here was developed in order to exploit the properties of the bifunctional reagent methyl-4-mercaptobutyrimidate. However, it is possible that under oxidative conditions disulfide bond formation may occur between cysteine residues present in the primary structures of unmodified proteins appropriately situated in the native ribosome structure. A number of ribosomal proteins contain cysteine (9, 10). An experiment similar to that described in Fig. 1c was performed with 30S subunits unmodified by reaction with methyl-4-mercaptobutyrimidate. The result is shown in Fig. 4, which shows a diagonal gel of ribosomal proteins oxidized in the 30S particle and in the first dimension, and then reduced in the second dimension. A number of protein

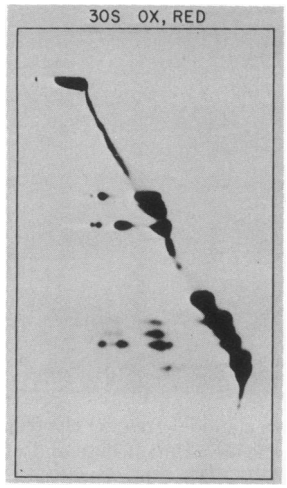

FIG. 4. Diagonal gel electrophoresis of 30S ribosomal proteins extracted from subunits modified only by oxidation, but not with methyl-4-mercaptobutyrimidate. The proteins were analyzed as described in Fig. 1c.

spots fall off the diagonal. These represent the components of disulfide linked dimers or oligomers formed between cysteine residues on proximal proteins in the 30S ribosomal subunit. All of the reduced components have not been identified at this time. Comparison of Fig. 4 with Fig. 1c indicates that proteins S4 and S13 are major crosslinked products linked by disulfide bond formation between the two unmodified proteins. This result indicates that regions of their structures are nearer than 30 A (the maximum distance that could be bridged by the crosslinking reagent). Other crosslinked protein pairs may also correspond to those formed with exogenous crosslinking reagents. On the other hand, it is readily apparent that many more disulfide-linked products are formed after the addition of new sulfhydryl groups to the ribosome. The formation of specific disulfide crosslinked protein pairs or oligomers due to oxidation may be related to the well-established observation that 30S ribosomes are more active in the presence of reducing agents (11).

The pattern of proteins having their origin in crosslinked products shown in Fig. 1c represents a characteristic "fingerprint" of the multiple protein–protein interactions within the 30S subunit. Crosslinking had previously been shown to be dependent on the use of intact particles as a substrate. No crosslinking takes place between extracted 30S proteins in solution (1). The same "fingerprint" of crosslinks is found whether disulfide bond formation is caused by treatment of the SH-charged ribosome (the particle after reaction with methyl-4-mercaptobutyrimidate) with hydrogen peroxide, air, or disulfide compounds of low molecular weight. The most noteworthy feature of the "fingerprint" of interactions among 30S ribosomal proteins presented here is that proteins of every molecular weight resolved by dodecyl sulfate gel electrophoresis are found in crosslinked material. Further analysis of these proteins by the techniques described here involving two-dimensional gel electrophoresis in urea indicates that every ribosomal protein can be found crosslinked to at least one other ribosomal protein. An unambiguous arrangement of all twenty-one 30S ribosomal proteins, based upon the identification of an interconnecting set of crosslinked dimers, should soon be possible.

This work was supported by a grant from the U.S. Public Health Service (GM-17924). Dr. Sommer is the recipient of a fellowship from the Swiss Cancer League. We thank Drs. William Benisek and John Hershey for their advice and critical reading of the manuscript.

1. Traut, R. R., Bollen, A., Sun, T. T., Hershey, J. W. B., Sundberg, J. & Pierce, L. R. (1973) *Biochemistry* **12**, 3266–3273.
2. Sun, T. T., Bollen, A., Kahan, L. & Traut, R. R. (1974) *Biochemistry* **13**, 2334–2340.
3. Kaltschmitt, E. & Wittmann, H. G. (1970) *Anal. Biochem.* **36**, 401–412.
4. Howard, G. A. & Traut, R. R. (1973) *FEBS Lett.* **29**, 177 181.
5. Hardy, S. J. S., Kurland, C. G., Voynow, P. & Mora, G. (1969) *Biochemistry* **8**, 2897–2905.
6. Lämmli, U. K. (1970 *Nature* **227**, 680–685.
7. Weber, K. & Kuser, D. J. (1971) *J. Biol. Chem.* **246**, 4504–4509.
8. Traut, R. R., Heimark, R., Sun, T. T., Hershey, J. W. B. & Bollen, A. (1974) in *The Ribosome*, eds. Nomura, M., Lengyel, P. & Tissieres, A. (Cold Spring Harbor, N. Y.) in press.
9. Acharya, A. S., & Moore, P. B. (1973) *J. Mol. Biol.* **76**, 207–221.
10. Ginzburg, I., Miskin, R. & Zamir, A. (1973) *J. Mol. Biol.* **79**, 481–494.
11. Zamir, A., Miskin, R. & Elson, D. (1971) *J. Mol. Biol.* **60**, 347–364.
12. Wittmann, H. G., Stöffler, G., Hindennach, I., Kurland, C. G., Randall-Hazelbauer, L., Birge, E. A., Nomura, M., Mizushima, S., Traut, R. R. & Bickle, T. A. (1971) *Mol. Gen. Genet.* **111**, 321–333.