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Disulfide Cross-Linking of *Escherichia coli* Ribosomal Proteins with 2-Iminothiolane (Methyl 4-Mercaptobutyrimidate): Evidence That the Cross-Linked Protein Pairs Are Formed in the Intact Ribosomal Subunit†

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ABSTRACT: The 30S ribosomal subunits of *Escherichia coli* were modified with 2-iminothiolane with the formation of amidine-linked sulfhydryl groups attached to ribosomal protein. The modified particle contained 61 sulfhydryl groups, 17-18 due to endogenous cysteine residues and the remainder from modification. The modified ribosomal subunits were oxidized to promote disulfide bond formation (cross-linking). About 15 free sulfhydryl groups per 30S particle remained after oxidation even when performed in the presence of 2-mercaptoethanol. Treatment of modified, oxidized particles with 4.0 M urea, 3.0 M LiCl exposed these sulfhydryl groups which reacted with iodoacetamide only after disruption of the native structure. The presence of these sulfhydryl groups prompted an investigation of possible sulfhydryl/disulfide interchange and random oxidation during extraction of cross-linked ribosomal proteins and/or the preparation of protein for diagonal polyacrylamide/dodecyl sulfate gel electrophoresis. Experiments were carried out to obtain direct evidence concerning the quantitative contribution of disulfide interchange and/or random oxidation during protein extraction to the pattern of cross-linked dimers previously reported. A radiolabeled cross-linked protein fraction of about 35 500 molecular weight was purified from cross-linked ^{35}S-labeled 30S subunits. The radiolabeled protein was added to nonradioactive cross-linked 30S ribosomal subunits immediately before extracting the protein under several different conditions including that with acetic acid used in earlier studies from this laboratory. The radioactivity was subsequently shown to migrate only at 35 500 molecular weight following analysis of the cross-linked protein by polyacrylamide/dodecyl sulfate gels. There was no evidence that disulfide interchange produced new cross-linked radioactive protein bands of different molecular weight. Similar results were obtained using the ^{35}S-labeled 30S ribosomal protein S4, which contains a single cysteine group. Radioactive S4 was found only as the monomeric protein. The results confirmed that earlier results on the cross-linking of the 30S ribosomal subunit reflect interprotein disulfide linked dimers formed in the intact particle. Random diagonal gel patterns have been deliberately formed by promoting oxidation of ribosomal proteins in solution. These patterns had no similarity with those previously published nor to those in which free sulfhydryl groups were blocked by alkylation. Additional evidence supports the conclusion that disulfide linked proteins reflect specific arrangements in the intact ribosome: cross-linked 30S subunits retained 60% of their activity in reassociation with 50S subunits and 30% in polyphenylalanine synthesis.

Chemical cross-linking of biological structures containing several polypeptide chains can provide useful information on the topography of the protein constituents. Such evidence is particularly valuable in the study of molecular structures for which X-ray crystallographic evidence is lacking or inadequate (Peters & Richards, 1977). A fundamental requirement is that the cross-linking reaction not perturb the native conformation of the biological structure. Cross-linking reagents based on bis(imido esters), such as dimethyl suberimidate (Davies & Stark, 1970), have been particularly useful since they react under mild conditions specifically with amino groups of proteins to yield amidine derivatives that retain positive charge (Hand & Jencks, 1962; Browne & Kent, 1975).

The topography of proteins of *Escherichia coli* ribosomal subunits has been extensively studied by a variety of experimental techniques in recent years (Brimacombe et al., 1976; Kurland, 1977a). Cross-linking studies using bis(imido esters) have been prominent in providing evidence on the spatial arrangement of ribosomal proteins (Bickle et al., 1972; Lutter

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et al., 1974a; Clegg & Hayes, 1974; Expert-Bezançon et al., 1977). Such reagents have also provided useful information about the location of binding sites of initiation factors to the ribosome (Bollen et al., 1975; Heimark et al., 1976). The products of cross-linking reactions have been principally analyzed using polyacrylamide/dodecyl sulfate gel electrophoresis in which cross-linked species were evident from their increased molecular weights. Identification of the protein components of cross-linked species can be made using immunological techniques (Lutter et al., 1974a; Heimark et al., 1976) or by cleavage of the cross-link (Bickle et al., 1972; Expert-Bezançon et al., 1977).

Analysis of cross-linked protein mixtures has been greatly facilitated by the development of reagents that contain within them an easily cleavable linkage (Traut et al., 1973; Lutter et al., 1974b; Coggins et al., 1976). Bifunctional imido esters that introduce a disulfide bond as the cross-link between different proteins have been found to be particularly useful compounds of this kind. The disulfide cross-linking reagent, 2-iminothiolane, has been especially productive in our investigation of ribosome protein topography (Sun et al., 1974; Sommer & Traut, 1974–1976; Kenny et al., 1975; Traut & Kenny, 1977; Jue et al., 1978). Other disulfide cross-linking reagents have been described (Perham & Thomas, 1971; Wang & Richards, 1974; Ruoho et al., 1975; Lomant & Fairbanks, 1976), and reversible disulfide cross-linking techniques have been used to study the arrangement of proteins in membranes (Wang & Richards, 1975; Ruoho et al., 1975), chromatin (Thomas & Kornberg, 1975), and viruses (Takemoto et al., 1977).

Kurland (1977b) and Peretz et al. (1976) have emphasized possible pitfalls in the use of disulfide cross-linking reagents arising from disulfide interchange or random oxidation during extraction and analysis of proteins from cross-linked ribosomal subunits. The wide use and apparent success of reagents such as 2-iminothiolane, dimethyl 3,3′-dithiobispropionimidate, and methyl 3-mercaptopropionimidate in the analysis of complex multi-protein structures (Sommer & Traut, 1976; Thomas & Kornberg, 1975; Peters & Richards, 1977) has prompted the more thorough investigation of the reliability of the method reported here. We have reinvestigated the possibility that disulfide interchange may have contributed to the cross-linked proteins reported previously (Sommer & Traut, 1974–1976). Patterns of cross-linked proteins obtained under conditions more rigorously designed to preclude disulfide interchange or random oxidation are indistinguishable from those published previously. Experiments which were specifically designed to detect random intermolecular cross-links gave no evidence for the occurrence of disulfide interchange or random oxidation during the analysis of proteins from oxidized ribosomal subunits. We conclude that the pairs of cross-linked proteins previously identified by Sommer & Traut (1974–1976) were indeed formed in the intact 30S ribosomal subunit. The modified methods for cross-linking with 2-iminothiolane are described in detail. They firmly establish the reliability of disulfide cross-linking and should extend the application of the technique to the ribosome and other protein assemblies.

Materials and Methods

Reagents. 2-Iminothiolane, formerly referred to as methyl 4-mercaptobutyrimidate, was purchased from Pierce and stored in a vacuum desiccator over Drierite at 4 °C. Urea "UltraPure" and 2-mercaptoethanol were obtained from Schwarz/Mann; iodoacetamide and 5,5′-dithiobis(2-nitrobenzoic acid)[1] were from Sigma; H_2O_2 AR 30% and LiCl were from Mallinckrodt Chemical Works. Triethanolamine was obtained from Eastman Organic Chemicals and was redistilled under vacuum before use. Acrylamide and bisacrylamide were obtained from Eastman and used without further recrystallization. Catalase (20 mg/mL, crystalline suspension) was purchased from Boehringer Mannheim GmbH. All other reagents were of analytical or reagent grade.

Radiochemicals. [^{35}S]Sulfate was purchased as carrier-free H_2SO_4 from ICN. [1-^{14}C]Iodoacetamide (specific radioactivity 57 mCi/mmol) was obtained from Amersham and diluted 1:100 with nonradioactive iodoacetamide before use.

The 30S Ribosomal Subunits. Ribosomal subunits were prepared from *E. coli* strain MRE600 grown in rich medium as described previously (Hershey et al., 1977). The 30S and 50S ribosomal subunits were purified by zonal centrifugation in 10 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, and 1 mM $MgCl_2$ (Eikenberry et al., 1970). Purified subunits were buffered with 10 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$ (TMA buffer) and stored at −70 °C. Radioactive ribosomal subunits were isolated from cells grown in the presence of [^{35}S]sulfate according to the method of Sun et al. (1974); the specific radioactivity of the ribosomal protein was about 12×10^7 cpm/mg.

Cross-Linking of the 30S Ribosomal Proteins with 2-Iminothiolane. The 30S ribosomal subunits (approximately 3 mg/mL) were treated with 2-iminothiolane (12 mM), in buffer (TEA–SH buffer) consisting of 50 mM triethanolamine hydrochloride, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, and 1% 2-mercaptoethanol as described previously (Traut et al., 1973; Sommer & Traut, 1975). Stock solutions of 2-iminothiolane (0.5 M; 68.5 mg/mL) were prepared immediately before use in a solvent consisting of equal volumes of 1.0 M triethanolamine hydrochloride, pH 8.0, and 1.0 M triethanolamine free base; the final pH was about 8.0. Ribosomal subunits were incubated with 2-iminothiolane for 20 min at 0 °C and dialyzed for 3 h at 4 °C against 100 vol of TEA–SH buffer without 2-mercaptoethanol (TEA buffer) with a buffer change each hour.

The modified ribosomal subunits were incubated with 40 mM H_2O_2 for 30 min at 0 °C, and then with 10 μg of catalase per mL of reaction mixture for 20 min at 0 °C to remove excess peroxide. These procedures have been shown to result in ribosomal particles reversibly cross-linked by disulfide bonds, many of which are between sulfhydryl groups on different ribosomal proteins and which link specific pairs of proteins into dimers (Sommer & Traut, 1974–1976).

Activity of 30S Subunits in Polyphenylalanine Synthesis. The 30S ribosomal subunits were assayed for poly(U)-dependent polyphenylalanine synthesis according to the method of Traut & Haenni (1967). Thirty-seven micrograms of 30S subunits in 10 μL of TEA buffer was mixed with 50 μL of 20 mM Tris-HCl, pH 7.2, 200 mM NH_4Cl, 20 mM $MgCl_2$, 25 mM dithioerythritol, and 2% 2-mercaptoethanol, and incubated for 20 min at 37 °C (Zamir et al., 1971). Oxidized (cross-linked) 30S subunits were also heat activated in the absence of reducing agents, following the catalase treatment to remove excess peroxide. The heat activation increased activity of unmodified 30S ribosomal subunits by 64% in these assays. Since the object of the assay was to assess the effect of oxidation on activity, reducing agents were omitted from the assay mixture. The reaction mixture, containing heat activated 30S subunits (37 μg) in a final volume of 200 μL, was incubated for 20 min at 37 °C, following addition of 190 μg of unmodified 50S ribosomal subunits in 10 μL of TMA buffer. Incorporation of L-[U-^{14}C]phenylalanine (specific radioac-

[1] Abbreviations used: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); bisacrylamide, *N,N′*-methylenebisacrylamide.

tivity 10 mCi/mmol) into trichloroacetic acid precipitable material was 4957 cpm/(min mg) unmodified (control) 30S subunits.

Reassociation of 30S Subunits into 70S Ribosomes. Reassociation of 30S subunits into 70S ribosomes was assayed following addition of 190 μg of unmodified 50S subunits (in 10 μL of TMA buffer) to 60 μg of 30S subunits in 100 μL of heat activation buffer at 0 °C. These samples were applied to sucrose density gradients (7–25%) prepared in 10 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, and 12 mM $MgCl_2$. Samples were centrifuged at 22 000 rpm for 11 h using a Beckman L5-65 ultracentrifuge with an SW-56 rotor at 4 °C. Gradients were scanned at 260 nm using a Gilford 2400-S recording spectrophotometer with a flow-cell attachment. Eighty-four percent of the unmodified (control) 30S subunits used in these experiments reassociated with unmodified 50S subunits to form 70S ribosomes under these conditions.

Extraction of Proteins from Cross-Linked 30S Ribosomal Subunits. Two methods were employed to extract ribosomal protein from the cross-linked ribosomal subunits following incubation with catalase. Ribosomal protein was extracted from cross-linked 30S subunits with 66% acetic acid according to Hardy et al. (1969), dialyzed against 6% acetic acid, and lyophilized. Alternatively the protein was extracted with LiCl/urea (Leboy et al., 1964); iodoacetamide (70 mM) was first added to the cross-linked ribosomal subunits in TEA buffer and after 30 min at 0 °C, 1 vol of 8.0 M urea, 6.0 M LiCl was added to give final concentrations of 35 mM iodoacetamide, 4.0 M urea, and 3.0 M LiCl. The solution was kept at 0 °C for 48 h and the RNA then removed by centrifugation in a Sorval SS-34 rotor at 12 000 rpm for 20 min. The protein solution was dialyzed against 6% acetic acid and lyophilized. Alternatively the protein solution was made 10% in trichloroacetic acid, incubated for 30 min at 0 °C, the protein precipitate collected by centrifugation, washed twice with a 1:1 mixture of ether and 95% ethanol, and dried under vacuum.

Polyacrylamide Gel Electrophoresis. Analysis of cross-linked ribosomal protein was performed using diagonal polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate using techniques that have been described in detail (Sommer & Traut, 1974–1976). Extracted samples of protein (about 400 μg) were dissolved in 60 μL of 100 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 6.0 M urea, and 15 mM iodoacetamide. Solutions were incubated at 25 °C for 30 min and heated to 65 °C for 10 min before application to gel tubes (10 × 0.3 cm) of 13.5% polyacrylamide with a stacking gel (1 × 0.3 cm) of 5% polyacrylamide (Laemmli, 1970). Electrophoresis was performed toward the anode at 2.0 mA per tube for 5 h. Gels were removed from the glass tubes and incubated in 25 mL of electrophoresis buffer, pH 8.8, containing 3% 2-mercaptoethanol as previously described (Sommer & Traut, 1976). After reduction of disulfide bonds for 10 min at 65 °C, the gels were incubated for 30 min at 25 °C in a similar buffer without 2-mercaptoethanol and adjusted to pH 6.8. Finally they were embedded at the origin of 13.5% polyacrylamide/sodium dodecyl sulfate gel slabs (25 × 13 × 0.3 cm). Electrophoresis was performed toward the anode at 90 V until the tracking dye (bromophenol blue) reached the bottom of the gel (about 20 h). The slabs were stained with Coomassie brilliant blue and destained as described previously (Sommer & Traut, 1974).

One-dimensional polyacrylamide/sodium dodecyl sulfate gel slabs (Laemmli, 1970) containing 13.5% polyacrylamide were used for the purification of specific ribosomal proteins, and were prepared and run under conditions similar to those described above. A 5% stacking gel of length of 3 cm was used. About 1.0 mg of ribosomal protein in 1.5 mL of solution containing 100 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 6.0 M urea, and 1% 2-mercaptoethanol was applied to a sample well 8-cm wide. Similar procedures were used to purify specific cross-linked protein species from 30S ribosomal protein derived from cross-linked subunits, except that the sample buffer contained 15 mM iodoacetamide and no 2-mercaptoethanol. After electrophoresis toward the anode at 90 V for 18 h, these gel slabs were stained with Amido black and destained as described previously (Sommer & Traut, 1974). Analysis of protein samples containing high salt concentration (3.0 M LiCl) was performed using similar 13.5% polyacrylamide gel slabs, with a 5% stacking gel of total length 5 cm and which contained sample wells with 200-μL capacity. Gels containing radioactive protein samples were stained, dried, and analyzed by radioautography (Howard & Traut, 1973). Kodak No-Screen Medical X-ray film was exposed for 2–4 weeks, depending upon the amount of radioactivity applied to the gel.

Ribosomal proteins were analyzed by two-dimensional polyacrylamide/urea gel electrophoresis using procedures described previously (Jue et al., 1978; Knopf et al., 1975). Radiolabeled proteins were mixed with nonradioactive total 30S ribosomal protein and were identified following radioautography of the stained gel dried onto filter paper as described above (Howard & Traut, 1973; Sommer & Traut, 1976).

Extraction of Protein from Polyacrylamide Gels. Polyacrylamide/dodecyl sulfate gel electrophoresis performed as described above was employed to purify radioactive S4, and a specific disulfide cross-linked dimer. The gel slab was stained with Amido black and the bands containing the protein(s) of interest were cut out, immersed in 100 mM Tris-acetate, pH 7.8, 1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol, pulverized, heated to 65 °C for 10 min, and kept at 25 °C overnight. Solid urea was added to 8.0 M and the dodecyl sulfate and stain were removed by passage through a column of Dowex AG1-X8 (20–50 mesh; Bio-Rad) in a modification of the procedure of Weber & Kuter (1971) described previously (Sommer & Traut, 1974). The protein sample was dialyzed against 6% acetic acid and lyophilized. In this way, protein S4 was purified from ^{35}S-labeled 30S ribosomal subunits with a yield of 1.3×10^6 cpm (approximately 11 μg). Similar procedures were used to purify a disulfide cross-linked protein fraction of about 35 500 molecular weight (mobility on polyacrylamide/dodecyl sulfate gels relative to 30S protein monomers of known molecular weight) from ^{35}S-labeled cross-linked 30S ribosomal subunits. In this case iodoacetamide (20 mM) was included in all buffers instead of 1% 2-mercaptoethanol. The estimated yield was 0.34×10^6 cpm (approximately 3 μg) of cross-linked protein fraction.

Estimation of Sulfhydryl Groups of Unmodified and Modified 30S Ribosomal Subunits. Samples of 30S ribosomal subunits (1.5 mg, 0.5 mL) were freed of 2-mercaptoethanol and reagents by gel filtration through Bio-Gel P-2 columns (100–200 mesh; 15.5 × 0.7 cm) equilibrated with oxygen-free, nitrogen-saturated 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 30 mM NH_4Cl, as described in the preceding paper (Jue et al., 1978). Titration of free sulfhydryl groups was performed with Nbs_2 as described previously (Jue et al., 1978).

Reaction of Unmodified and Modified 30S Ribosomal Subunits with [1-^{14}C]Iodoacetamide in 4.0 M Urea, 3.0 M LiCl. 2-Mercaptoethanol and reagents were removed from samples of 30S ribosomal subunits by gel filtration. Alkylation of the resulting samples of 30S ribosomal subunits (2.0 mg/mL) was performed by the addition of 1 vol of 8.0 M urea, 6.0 M LiCl, 0.2 M Tris-HCl, pH 8.0, containing [1-^{14}C]iodoacetamide (specific radioactivity 0.57 mCi/mmol), as de-

scribed previously (Jue et al., 1978). Samples (75 μL) were mixed with 2.5 mL of 5% trichloroacetic acid containing 2.5% 2-mercaptoethanol and incubated for 30 min at 94 °C. The precipitated 30S ribosomal protein was filtered through glass-fiber discs (Whatman, GF/C), washed thoroughly with 5% trichloroacetic acid, dried, and counted in a Beckman LS-200B liquid scintillation counter in 5.0 mL of toluene containing 2,5-diphenyloxazole (4 mg/mL), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.05 mg/mL). The counting efficiency was 89%.

Results

Oxidation of 30S Ribosomal Subunits Modified with 2-Iminothiolane. Oxidation of ribosomal subunits modified with 2-iminothiolane leads to the formation of intermolecular and very likely of intramolecular disulfide bonds. Experiments were performed to investigate whether the oxidative cross-linking reaction led to the complete disappearance of free ribosomal sulfhydryl groups.

30S ribosomal subunits (3 mg/mL) were incubated with 2-iminothiolane (12 mM) in TEA–SH buffer for 20 min at 0 °C. The solution was then dialyzed for 3 h against 100 vol of TEA buffer at 4 °C. This procedure followed that used previously in cross-linking studies of the 30S subunit (Sommer & Traut, 1974–1976). Figure 1 shows the presence of 61 sulfhydryl groups per 30S ribosomal subunit. Since the unmodified subunit was found to contain 17–18 sulfhydryl groups, it was clear that 43 were due to modification with 2-iminothiolane. The effect of oxidation with 40 mM H_2O_2 for 30 min at 0 °C on the number of sulfhydryl groups of both unmodified and modified 30S ribosomal subunits was examined (Figure 1). About 15 sulfhydryl groups could be titrated by Nbs_2 in both the unmodified and modified subunits (Figure 1) and were apparently resistant to oxidation. These 15 sulfhydryl groups remained even when higher concentrations of H_2O_2 were employed (160 mM) or when oxidation was performed in the presence of 2-mercaptoethanol (7 mM) added to promote the formation of mixed disulfides with the mercaptan. The 15 resistant sulfhydryl groups in unmodified subunits became reactive with Nbs_2 only when the 30S particles were denatured with dodecyl sulfate (Figure 1). Since the number of resistant sulfhydryl groups was the same for unmodified and modified particles, it was concluded that they represented the endogenous cysteine residues rather than those of the lysine derivatives formed by modification with 2-iminothiolane. Figure 1 shows that the sulfhydryl groups introduced by modification with 2-iminothiolane reacted rapidly with Nbs_2. The rapidly titratable sulfhydryl groups were more readily oxidized and were susceptible to oxidation at low H_2O_2 concentration (result not shown). These experiments showed that free sulfhydryl groups remained in the disulfide cross-linked ribosomal subunits after exhaustive oxidation, and strongly suggested that they corresponded to endogenous cysteine residues which were relatively inaccessible either to the sulfhydryl reagent or to oxidation.

Polyacrylamide/Dodecyl Sulfate Diagonal Gel Electrophoresis of Cross-linked 30S Ribosomal Protein Prepared by Extraction with Acetic Acid. The diagonal gel electrophoretic pattern of protein extracted from cross-linked 30S ribosomal subunits using the acetic acid procedure (Hardy et al., 1969) is shown in Figure 2A. This pattern is similar, nearly identical, to those published previously using identical techniques (Sommer & Traut, 1974, 1975). This procedure included the following steps: (1) modification with 2-iminothiolane; (2) oxidation with H_2O_2; (3) removal of excess peroxide by addition of catalase; (4) extraction of protein with

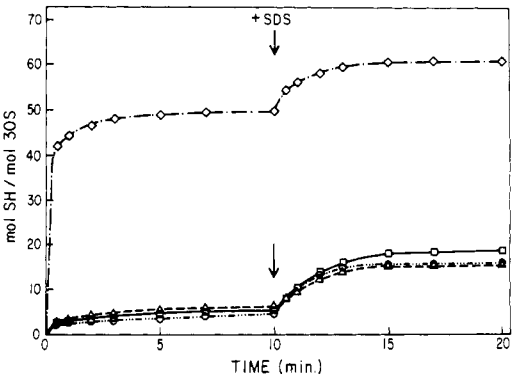

FIGURE 1: Determination of sulfhydryl groups of 30S ribosomal subunits. Samples of unmodified 30S ribosomal subunits (□—□) and modified 30S subunits (◇—·—◇) were assayed. The 30S subunits (3 mg/mL in TEA–SH buffer) were modified by treatment with 2-iminothiolane (12 mM) for 20 min at 0 °C followed by dialysis for 3 h against 100 vol of TEA buffer with a buffer change each hour. Sulfhydryl groups also were assayed following oxidation of samples of unmodified 30S ribosomal subunits (△———△) and modified 30S subunits (○—···—○) with 40 mM H_2O_2 for 30 min at 0 °C. Following gel filtration of each sample as described in the text, sulfhydryl groups were assayed at 25 °C with Nbs_2, measuring the absorbance change at 412 nm. Sodium dodecyl sulfate (0.9%) was added after 10 min.

66% acetic acid; (5) dialysis of the RNA-free protein against 6% acetic acid; (6) lyophilization; (7) diagonal gel electrophoresis. It is possible to match all the cross-linked pairs of proteins that have been identified previously with regions of stained protein below the diagonal. Nonetheless, the results of the Nbs_2 titrations which showed that sulfhydryl groups remain after oxidative cross-linking led us to design further experiments to exclude more rigorously the possibility that these free sulfhydryl groups could contribute towards random oxidation or disulfide interchange. In the earliest studies (Sommer & Traut, 1974), no attempts were made to block these groups on the assumption that under the conditions of acid extraction possible residual sulfhydryl groups would be unreactive and that disulfide interchange would not occur to a significant extent (Eldjarn & Pihl, 1957a; Spackman et al., 1960).

After 3 h of dialysis employed to reduce the 2-mercaptoethanol concentration before oxidation of the modified ribosomal subunits, a significant amount of 2-mercaptoethanol (0.5–1.0 mM) still remained as determined by Nbs_2 titration (Jue et al., 1978); the modified ribosomal subunit was still in a reducing environment until oxidation by H_2O_2. Total removal of 2-mercaptoethanol employing Sephadex G-25 columns or addition of 2-mercaptoethanol (7 mM) before oxidation by peroxide had no effect on the apparent yield or pattern of cross-linked protein shown by diagonal polyacrylamide gel electrophoresis (results not shown): diagonal patterns were identical with that shown in Figure 2A. Thus possible formation of mixed disulfides between protein and 2-mercaptoethanol did not have any detectable effect on the specificity or yield of inter-protein disulfide formation.

The diagonal gel pattern of proteins prepared by acetic acid extraction of cross-linked 30S ribosomal subunits, performed as in Figure 2A but without addition of catalase to remove the excess H_2O_2, is shown in Figure 2B. The acidic solution of extracted protein would still contain peroxide which could promote oxidation of newly exposed free sulfhydryl groups or contribute to disulfide interchange. Inspection of Figure 2B shows that the pattern below the diagonal, corresponding to cross-linked protein, was similar to that of panel A (Figure 2).

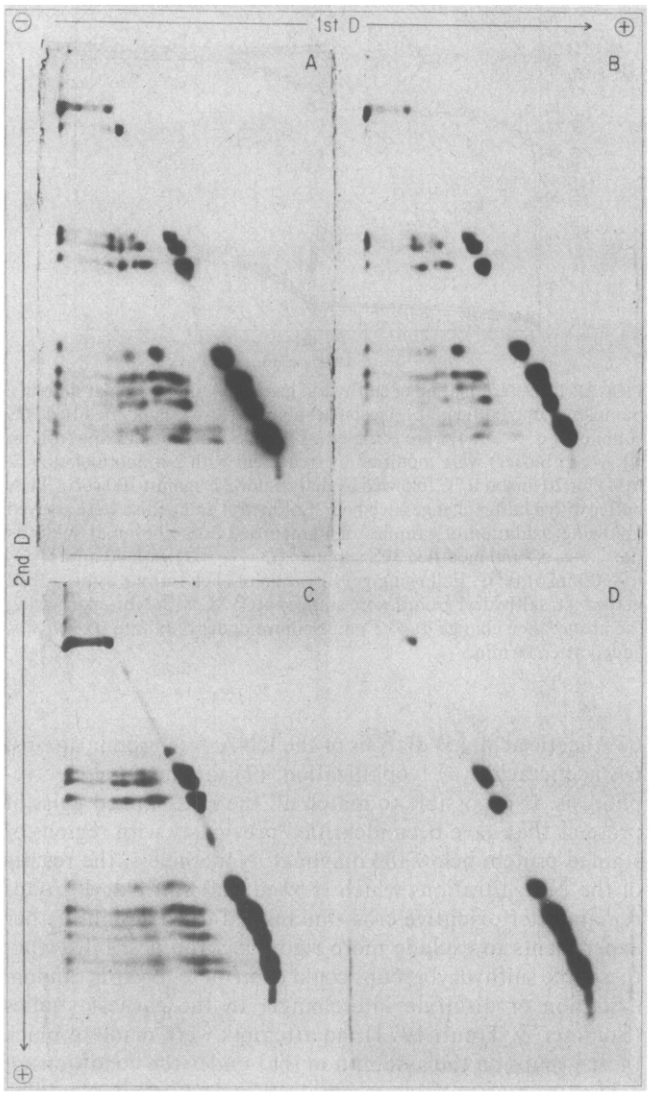

FIGURE 2: Effect of H_2O_2 and catalase on the diagonal gel electrophoretic pattern of protein extracted from amidinated 30S ribosomal subunits. (Panel A) Protein from intact 30S ribosomal subunits modified with 2-iminothiolane (12 mM), oxidized with H_2O_2 (40 mM), and then incubated with catalase (10 μg/mL) using procedures described in Materials and Methods. (Panel B) Protein from 30S ribosomal subunits treated exactly as for panel A, only without the incubation with catalase. (Panel C) Protein from 30S subunits modified with 2-iminothiolane and extracted without addition or either H_2O_2 or catalase. (Panel D) Protein from 30S subunits modified with 2-iminiothiolane and then incubated at 65 °C for 10 min in the presence of 2% sodium dodecyl sulfate before treatment with 40 mM H_2O_2 at 30 °C for 20 min. The proteins in every case were extracted with 66% acetic acid.

Randomization of Diagonal Gel Pattern by Oxidation in Acetic Acid. The diagonal gel pattern shown in Figures 2A and B is highly reproducible. It represents a distinctive specific array of spots beneath the diagonal. Individual spots are characterized by differences in position and in intensity. Such a pattern would not be expected to arise from random interactions between proteins. To establish this point an experiment was designed to create a "random" diagonal pattern. The reduced modified ribosomal subunit was extracted with acetic acid prior to intentional oxidation, so that the sulfhydryl-rich protein would be subjected to air oxidation during extraction, dialysis against 6% acetic acid, and lyophilization. Figure 2C shows that the modified ribosomal protein had undergone some oxidation as indicated by the appearance of stained material below the diagonal. This oxidation during extraction of protein and preparation of the sample for electrophoresis produced a

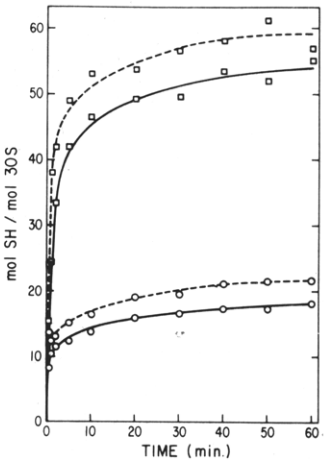

FIGURE 3: Reaction of 30S ribosomal subunits with [1-^{14}C]iodoacetamide. Unmodified 30S subunits (○), and 30S ribosomal subunits modified with 2-iminothiolane (12 mM) as described in Figure 1 (□), were alkylated. The 30S subunits (1 mg/mL) were incubated at 25 °C in 4.0 M urea, 3.0 M LiCl, 100 mM Tris-HCl, pH 8.0, and either 20 mM iodoacetamide (solid lines) or 40 mM iodoacetamide (dashed lines). The extent of reaction was determined by precipitating the protein with trichloroacetic acid, and counting the protein-bound radioactivity as described in the text.

gel pattern distinctly different from that derived from the oxidation of intact modified particles. Patterns similar to Figure 2C were also obtained following addition of H_2O_2 after the extraction of proteins from reduced modified subunits with acetic acid.

Figure 2D shows the diagonal gel pattern obtained when modified 30S subunits were extracted directly in dodecyl sulfate and then oxidized with H_2O_2. The solution of protein in dodecyl sulfate was then adjusted to 66% acetic acid to precipitate the RNA and was dialyzed and lyophilized prior to electrophoresis. No inter-protein cross-linking occurred as indicated by the absence of spots below the diagonal. The oxidation represented by Figure 2C must take place prior to resuspension of proteins in dodecyl sulfate for electrophoresis. It appeared that the modified ribosomal proteins, having bound dodecyl sulfate, behaved as a mixture of monomeric proteins toward cross-linking reactions owing to negative charge interactions which would keep the polypeptide chains separate from each other. The sample of protein used in Figure 2D was assayed for sulfhydryl groups; none were found. This indicated the formation of intra-protein disulfide bonds, and/or the formation of mixed disulfides between protein and the remaining 2-mercaptoethanol, upon oxidation in the presence of dodecyl sulfate. There was no evidence for any disulfide interchange leading to inter-protein cross-links.

Alkylation of Sulfhydryl Groups of Unmodified and Modified 30S Ribosomal Subunits. The demonstration of free sulfhydryl groups in the cross-linked protein prompted an investigation of the effect on the diagonal pattern of blocking these groups. The conditions of acetic acid extraction (Hardy et al., 1969) preclude the blocking of sulfhydryl groups with alkylating reagents such as iodoacetamide. Therefore, it was decided to employ the LiCl/urea method for extracting ribosomal protein (Leboy et al., 1964), in which the pH, adjusted to 8.0, allows rapid alkylation of sulfhydryl groups.

The addition of 4.0 M urea, 3.0 M LiCl to 30S ribosomal subunits exposed the inaccessible sulfhydryl groups for reaction with Nbs_2 at a rate comparable to that of 1% dodecyl sulfate (result not shown). When samples of unmodified or modified 30S subunits (2 mg/mL) were mixed with 1 vol of 8.0 M urea, 6.0 M LiCl, 0.2 M Tris-HCl, pH 8.0, containing [1-^{14}C]io-

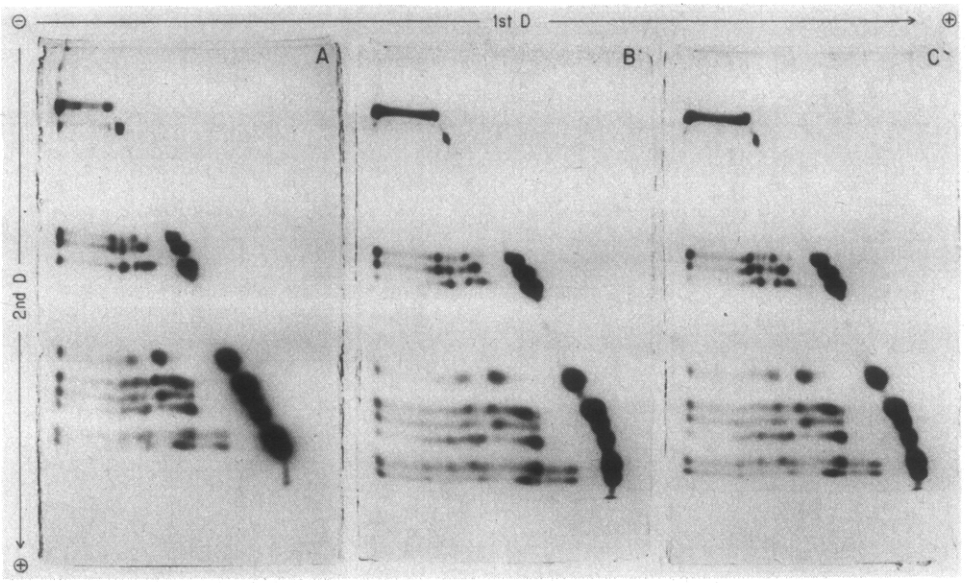

FIGURE 4: Comparison of different methods of protein extraction by diagonal polyacrylamide/dodecyl sulfate gel electrophoresis. Protein was extracted from cross-linked 30S subunits as follows: (Panel A) The 66% acetic acid method of Hardy et al. (1969), and then dialysis against 6% acetic acid and lyophilization. (Panel B) The iodoacetamide/LiCl/urea method, and then dialysis against 6% acetic acid and lyophilization. (Panel C) The iodoacetamide/LiCl/urea method, and then trichloroacetic acid precipitation for recovery of protein.

doacetamide, there was rapid alkylation of the sulfhydryl groups indicated by the increase in protein-bound radioactivity, shown in Figure 3. Eighty-five percent of the sulfhydryl groups of modified 30S subunits were alkylated within 10 min. The incorporation of radioactive iodoacetamide was in good agreement with the number of sulfhydryl groups assayed by Nbs_2. Thus LiCl/urea extraction of ribosomal proteins from RNA offered the possibility of rapidly blocking with iodoacetamide the sulfhydryl groups remaining after disulfide cross-linking of the 30S particle.

Diagonal Gel Electrophoretic Patterns of Proteins Extracted with LiCl/Urea or Acetic Acid. Protein from cross-linked 30S ribosomal subunits was prepared both by extraction with LiCl/urea in the presence of iodoacetamide and by extraction with acetic acid. The protein extracted with LiCl/urea was recovered both by dialysis and lyophilization, and by precipitation with trichloroacetic acid. The latter provided a faster method for recovery of protein. The diagonal gel patterns were compared and the results, shown in Figure 4, were very similar. If air oxidation or disulfide interchange occurred in acetic acid, the yields must be so low and the products so diverse that the characteristic pattern was not perturbed. The evidence supports the view that the yield of specific cross-linked protein pairs resulting from random oxidation or disulfide interchange is so low as to be undetectable on diagonal gels and does not perturb the characteristic pattern which results from oxidation (disulfide cross-linking) of the intact particle. All cross-linked pairs of proteins previously identified correspond to spots visible in each panel of Figure 4.

Radiolabeled S4 and S5–S8 as Probes for Possible Interchange. The possibility of disulfide interchange during the preparation of protein samples from cross-linked 30S subunits was examined more directly by experiments in which (1) a pure radioactive ribosomal protein containing a free sulfhydryl group and (2) a radioactively labeled cross-linked dimer were mixed with nonradioactive cross-linked ribosomal protein extracted from 30S subunits. Disulfide bond formation (1) or interchange (1 and 2) during preparation of the protein samples for electrophoresis would lead to the appearance of radioactive protein in regions of gel electropherographs different from those corresponding to the original radioactive species.

Protein S4, which has a single cysteine residue (Reinbolt & Schiltz, 1973), was purified from unmodified ^{35}S-labeled 30S ribosomal subunits by elution from a polyacrylamide/dodecyl sulfate gel shown in Figure 5A. Sulfhydryl determination with Nbs_2 confirmed the presence of the sulfhydryl group. S4 was lyophilized and resuspended in the absence of reducing or alkylating agents and a small amount was found to be present as a dimer which could be converted to S4 upon mild reduction. Radioactively labeled proteins were extracted from 30S ribosomal subunits that had been cross-linked under standard conditions. Figure 5B shows the electrophoretic pattern of these proteins. One very prominent cross-linked fraction, indicated in Figure 5B, was eluted. Polyacrylamide/dodecyl sulfate gel electrophoresis under nonreducing conditions showed all the radioactivity to migrate as a band of unique molecular weight: 35 500 relative to the mobility of 30S protein markers. Two-dimensional polyacrylamide/urea gel electrophoresis under reducing conditions in the presence of 30S protein marker showed most of the radioactivity to coincide with proteins S5 and S8 indicating that the dimer isolated was S5–S8.

Nonradioactive 30S ribosomal subunits (5.5 mg/mL) were modified with 2-iminothiolane, oxidized with H_2O_2, incubated with catalase, and divided into several aliquots (330 μg) each of which was extracted, mixed with the radioactive probe, and prepared for gel electrophoresis according to Scheme I. Lyophilized radiolabeled protein (110 000 cpm of S4; 60 000 cpm of cross-linked dimer) was dissolved in 8.0 M urea, 6.0 M LiCl (400 μL), and aliquots (65 μL) were added to the nonradioactive cross-linked subunits while initiating one of the different methods of protein extraction. In the case of the samples of cross-linked 30S ribosomal subunits prepared for acetic acid extraction (samples 5 and 6, Scheme I), glacial acetic acid (to 66%) and $MgCl_2$ (to 34 mM) were added simultaneously with the aliquot of radiolabeled protein dissolved in 8.0 M urea, 6.0 M LiCl.

Following centrifugation to remove RNA, the protein from each sample was recovered by methods shown in Scheme I. The samples were then prepared for electrophoresis on one-dimensional polyacrylamide/dodecyl sulfate gel slabs by the addition of sodium dodecyl sulfate (2%) to the resuspended protein, as described in the legend to Figure 6.

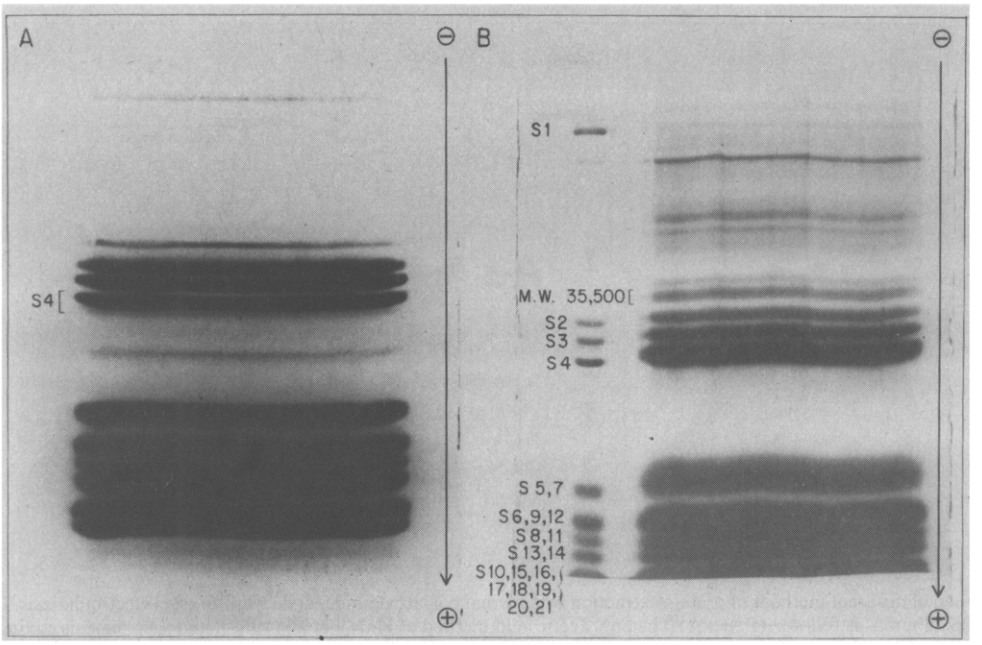

FIGURE 5: Isolation of ^{35}S-labeled proteins from polyacrylamide/dodecyl sulfate gel slabs. (Panel A) The stained pattern of reduced protein from unmodified 30S subunits (3 mg) from cells grown in the presence of [^{35}S]sulfate. Protein S4 was cut out and purified from this gel as described in Materials and Methods. (Panel B) The stained pattern of protein from 30S subunits (3 mg) that had been modified by 2-iminothiolane and oxidized as described in Materials and Methods, to produce high molecular weight cross-links. A prominent cross-linked protein fraction of 35 500 molecular weight (mobility relative to markers of 30S proteins of known molecular weight) was cut out and purified from this gel as described in the text.

SCHEME I: Experiment to Detect Random Oxidation and/or Disulfide Interchange during Protein Extraction from Cross-Linked 30S Ribosomal Subunits.[a]

30S Ribosomal Subunits
Modification
Oxidation (cross-linking)
Catalase Addition
sample

	1	2	3	4	5	6
Iodoacetamide (70 mM)	+	–	+	+	–	–
LiCl/urea extraction	+	+	+	+	–	–
66% acetic acid extraction	–	–	–	–	+	+
Treatment of protein following removal of RNA by centrifugation	Precipitate with 10% trichloro-acetic acid	Dialyze against 6% acetic; lyophilize	Dialyze against 6% acetic; lyophilize	Store at −70 °C	Dialyze against 6% acetic; lyophilize	Dialyze against 6% acetic; lyophilize

[a] Outline of sample preparation in which either [^{35}S] S4 or [^{35}S] S5-S8 was mixed with nonradioactive cross-linked 30S ribosomal subunits. The radioactive probes dissolved in 8.0 M urea, 6.0 M LiCl were added simultaneously with the initiation of extraction of protein from ribosomal RNA. The resulting samples of protein were analyzed by electrophoresis on one-dimensional polyacrylamide/dodecyl sulfate gel slabs, shown in Figure 6;

The stained patterns of protein bands are shown in Figure 6A. All samples which had been prepared by treatment with LiCl/urea in the presence of iodoacetamide (samples 1, 3, and 4) were indistinguishable. A slight loss of definition of cross-linked protein bands was observed for sample 2, the sample extracted with LiCl/urea in the absence of iodoacetamide. Acetic acid extraction of ribosomal protein resulted in some loss of resolution of the pattern of cross-linked proteins of molecular weight greater than 55 000 (sample 5). Sample 6 shows protein which was not oxidized in the particle but in which the material of elevated molecular weight was due to air oxidation during acetic acid extraction.

Panels B and C (Figure 6) show radioautographs of gels like that shown in panel A to which either [^{35}S]S4 or [^{35}S]S5–S8 had been added. S4, the S4 dimer and S5–S8 migrated in their expected positions. If some amount of the radioactive probes had reacted with nonradioactive protein, then they must have reacted to form small amounts of so many different products that they were not detectable. In no case, even when mixed with sulfhydryl-rich protein undergoing random oxidation as in sample 6, did radioactivity appear in new regions of the gel.

Activity of Cross-Linked 30S Subunits. Cross-linked 30S subunits were assayed with control 50S subunits for activity in poly(U)-directed polyphenylalanine synthesis. The effects of modification and oxidation were assayed separately and together (cross-linked). The results are given in Table I. Both

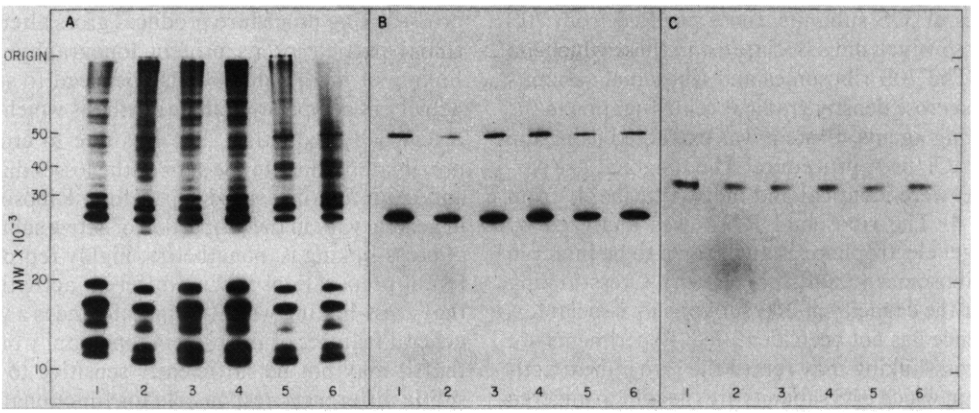

FIGURE 6: One-dimensional polyacrylamide/dodecyl sulfate gel electrophoretic analysis of proteins extracted from cross-linked 30S ribosomal subunits. The proteins of samples 1 → 6 shown in panel A were extracted from the cross-linked 30S particle using several different procedures outlined in Scheme I. The proteins of samples 1, 2, 3, 5, and 6 were resuspended in a solvent (130 μL) consisting of equal volumes of TEA buffer, and 8.0 M urea, 6.0 M LiCl, and containing 35 mM iodoacetamide. The proteins of sample 4 were already in such a solvent (Scheme I). Each sample was incubated with 2% sodium dodecyl sulfate at 65 °C for 10 min prior to application to the polyacrylamide gel. Electrophoresis was performed toward the anode as described in Materials and Methods. When extraction of protein from nonradioactive cross-linked 30S ribosomal subunits was initiated, the cross-linked protein was mixed with either radioactive protein S4, or a radioactive cross-linked dimer. (Panel A) Pattern of protein from samples 1 → 6 (Scheme I) stained with Coomassie brilliant blue. (Panel B) Radioautograph showing samples 1 → 6 (panel A) which had been mixed with radioactive protein S4 during extraction of protein. (Panel C) Radioautograph showing samples 1 → 6 (panel A) which had been mixed with a radioactive cross-linked dimer during extraction of protein.

TABLE I: Effect of Modification and Oxidation on the Activity of 30S Ribosomal Subunits in Poly(U)-Directed Polyphenylalanine Synthesis.[a]

30S subunits	reducing agents	polyphenylalanine synthesis (%)
unmodified (control)	+	100
unmodified, oxidized	−	75 ± 3
unmodified, oxidized	+	80 ± 7
modified	+	64 ● 5
modified, oxidized	−	29 ± 2
modified, oxidized	+	40 ± 6

[a] The 30S subunits were modified with 2-iminothiolane and/or oxidized with H_2O_2 as described in Materials and Methods. Heat activation, in the presence or absence of reducing agents, and the assay were carried out as described in Materials and Methods. These results were the mean of five separate experiments.

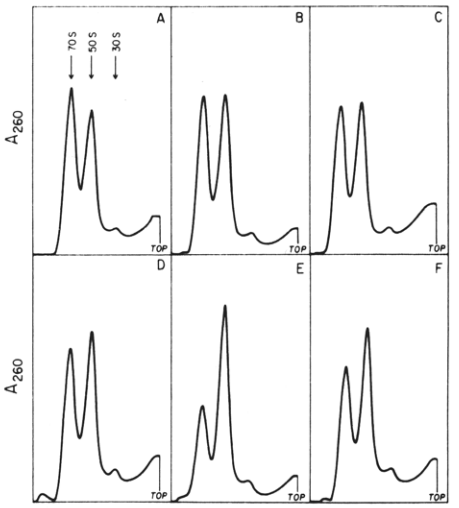

FIGURE 7: Sucrose density gradient analysis of the association of unmodified, modified, and oxidized 30S subunits with unmodified 50S subunits to form 70S ribosomes. All samples of 30S subunits were heat activated for 20 min at 37 °C under conditions described in Materials and Methods. Gradient analysis was performed as described in Materials and Methods with the following samples, to which was added 190 μg of 50S subunits prior to centrifugation: (panel A) 60 μg of unmodified 30S subunits heat activated in the presence of reducing agents; (panel B) 60 μg of unmodified 30S subunits oxidized with H_2O_2 and heat activated in the absence of reducing agents; (panel C) identical with panel B except that heat activation was in the presence of reducing agents; (panel D) 60 μg of modified 30S subunits heat activated in the presence of reducing agents; (panel E) 60 μg of modified 30S subunits oxidized with H_2O_2 and heat activated in the absence of reducing agents; (panel F) identical with panel E except that heat activation was in the presence of reducing agents. The base line was high and variable in the region of the small 30S peak owing to the large excess of 50S over 30S and to the absorbance at 260 nm of small molecular weight materials such as 2-mercaptoethanol. Therefore, the small 30S peak was not quantified. The results discussed in the text are derived from quantification of the 70S peaks.

oxidation and modification independently caused partial losses in activity, 25% and 35%, respectively. Cross-linked 30S subunits showed a 70% loss of activity assayed in the absence of reducing agent and 60% loss of activity in its presence. The loss of activity in protein synthesis cannot be correlated with the effect on subunit association since the latter was reversed by reduction (Figure 7).

Effect of Cross-Linking on the Formation of 70S Ribosomes. The yield of intermolecular disulfide cross-links, estimated visually, is low and likely does not exceed 20%. Two competing reactions limit the yield of dimers: the formation of higher oligomers and the formation of intramolecular disulfide bonds. Nonetheless, experiments were designed to exclude the possibility that all the cross-links were formed in a subpopulation of damaged or abnormal particles. Earlier experiments had shown that cross-linked and control 30S subunits cosedimented in sucrose gradient centrifugation (Traut et al., 1973; Sun et al., 1974). The experiment shown in Figure 7 tested the effect of oxidation and cross-linking of unmodified and modified 30S subunits on their ability to associate with 50S subunits to form 70S ribosomes. Comparison of Figure 7, panel B, with panel A, shows that oxidation of unmodified subunits caused a 10% loss in association which was not reversed by reduction (panel C). Modification without oxidation had little effect (Figure 7, panel D). Oxidation (cross-linking) of modified subunits lowered association by about 40% (panel E), and this loss was reversed by reduction (panel F).

The possibility that cross-linking was uniquely responsible for the failure of 40% of the subunits to associate with 50S subunits was evaluated by comparing the diagonal gel patterns

of the two classes of 30S subunits: those purified from 70S particles (i.e., those which did associate); and those which did not reassociate. The 70S ribosomes and ribosomal subunits were purified by sucrose density gradient centrifugation in the absence of reducing agents. Protein was extracted using the iodoacetamide/LiCl/urea procedure. The diagonal gel patterns (not shown) were identical and indistinguishable from those in Figure 4. The ribosomal RNA was analyzed by polyacrylamide gel electrophoresis and shown to be intact in both classes of ribosomes (results not shown). Cross-linking appears to impair the capacity of 30S subunits to associate. A structural difference has not been identified. Experiments not shown suggest cross-linking may retard the rate of heat activation, a reaction in which 30S subunits are thought to undergo a conformational change promoting association (Zamir et al., 1971; Hapke & Noll, 1976).

Discussion

Reversible cross-linking with 2-iminothiolane has been used for several years in this laboratory to define the protein topography of ribosomal subunits from *E. coli*. The purpose of this report has been to bring together a variety of experiments which substantiate the view that the procedure leads to the identification of cross-linked protein dimers which are indicative of spatial relationships between proteins in the intact ribosomal subunit in its original conformation. The experiments were addressed primarily to two questions. Does the cross-linking reaction significantly perturb the conformation of the ribosome or detect relationships in an already perturbed subpopulation of ribosomal subunits? Does disulfide interchange or random oxidation take place and lead to the identification of cross-linked dimers not representative of the protein topography of the subunit? Extensive evidence has been accumulated on ribosomal protein topography based on reversible disulfide cross-linking. The method has potential wide applicability in other systems. However, it has been criticized as being open to possible artifacts as discussed here (Peretz et al., 1976; Kurland, 1977b). For this reason, it was decided to perform experiments to evaluate as thoroughly as possible the contribution of random cross-linking in the formation and identification of dimers between ribosomal proteins. The results show that reversible disulfide cross-linking performed as described provides reliable information on protein proximities in the intact ribosomal particle.

The cross-linking procedure used in this laboratory involves three steps: (1) modification of the ribosomal subunit with 2-iminothiolane to introduce additional sulfhydryl groups; (2) oxidation of the modified particle to promote disulfide bond formation; (3) extraction of the protein and analysis by two-dimensional (diagonal) polyacrylamide/dodecyl sulfate gel electrophoresis. Early experiments (Traut et al., 1973; Sun et al., 1974; Sommer & Traut, 1974) showed that sedimentation velocity of 30S subunits was not changed by cross-linking. The results presented here showed that 60% of cross-linked 30S subunits reassociate with 50S subunits to form 70S particles. The reassociated cross-linked 30S subunits were obtained from 70S ribosomes and compared by diagonal gel electrophoresis with the 40% which did not reassociate. The patterns of cross-linked proteins were indistinguishable. It has also been shown that the 16S RNA remains intact following cross-linking (results not shown). Furthermore, the majority of oxidized 30S ribosomal subunits appear normal when viewed in the electron microscope, with no more than 7% more abnormal images than present in control preparations (James Lake, personal communication).

On the basis of these results it appears unlikely that the cross-linking procedure produces gross alteration of the ribosomal particle or its protein topography. However, cross-linking of modified subunits does lead to substantial loss of activity in polyphenylalanine synthesis which is not completely reversed by reduction. The loss due to cross-linking is approximately equal to the sum of the losses due to modification and oxidation measured separately. Ribosome preparations in general vary in the proportion of active subunits. The pattern of cross-linking is, nonetheless, highly reproducible with different preparations having different activities. We conclude that cross-linking with 2-iminothiolane is a valid probe of the general features of the protein topography of the particle, but that it may not be sufficiently sensitive to detect the more subtle differences responsible for functional heterogeneity.

The second question, that of possible disulfide interchange or random oxidation, was considered in the initial work using reversible disulfide cross-linking with 2-iminothiolane (Traut et al., 1973). The conclusion that neither occurred was based on observations that one-dimensional polyacrylamide/dodecyl sulfate gel patterns were similar using oxidation or bis(phenylmaleimides) to cross-link 30S ribosomal subunits modified with 2-iminothiolane. The gel patterns were also similar to those obtained with dimethyl suberimidate. However, the resolution of one-dimensional electrophoresis is low compared with that of the two-dimensional diagonal gel electrophoresis employed in later investigations. This consideration prompted, in part, the reinvestigation of the problem which is reported here. More important was the observation that free sulfhydryl groups, possible catalysts of interchange or substrates for random oxidation, remained following oxidation of ribosomal subunits modified with 2-iminothiolane. This result was in contrast to that of Thomas & Kornberg (1975) who found that there were no free sulfhydryl groups following oxidation of histone octamers modified with methyl 3-mercaptopropionimidate, a reagent similar to that used in the experiments reported here.

Sulfhydryl groups are readily oxidized under mild conditions to form disulfides which are resistant to further oxidation (Jocelyn, 1972). Sulfhydryl oxidation is pH dependent, with the rate of oxidation increasing with increasing pH, implicating the sulfhydryl anion in the reaction mechanism (Jocelyn, 1972). This is also true for the oxidation of sulfhydryl groups by H_2O_2 (Pascal & Tarbell, 1957), the oxidizing agent used in the cross-linking studies of Sommer & Traut (1974–1976). The evidence of Figures 1 and 2 shows that about 15 free sulfhydryl groups remain unoxidized after peroxide treatment of the modified ribosomal subunit. These are likely to be sulfhydryl groups of cysteine residues of the ribosomal proteins. Acidification upon protein extraction should prevent further oxidation of these free sulfhydryl groups (Jocelyn, 1972). However, the rate of oxidation of sulfhydryl groups by H_2O_2 may be still significant even at pH 2.0 (Pascal & Tarbell, 1957; Jocelyn, 1972), suggesting that the nonionized sulfhydryl group may also participate in the mechanism of oxidation by peroxide. Therefore, it is essential to remove excess H_2O_2 by addition of catalase before extracting the proteins to ensure that there is no contribution to the pattern of disulfide cross-linked proteins due to sulfhydryl oxidation occurring during acid extraction of the proteins (Pascal & Tarbell, 1957). Disulfide/disulfide interchange may also occur under acidic conditions in the presence of H_2O_2 (Benesch & Benesch, 1958). The addition of catalase was used in all but the earliest studies of Sommer & Traut (1974), although this was not emphasized until recently (Sommer & Traut, 1976).

It was assumed in the initial experiments that extraction of ribosomal proteins in 66% acetic acid would prevent disulfide

interchange. The assumption was based upon results showing that disulfide/disulfide interchange reactions are catalyzed by free sulfhydryl groups which take part in sulfhydryl/disulfide exchange reactions (Ryle & Sanger, 1955; Jocelyn, 1972). The sulfhydryl/disulfide exchange reaction is strongly pH dependent with the rate of reaction being proportional to the concentration of the sulfhydryl anion (Eldjarn & Pihl, 1957a). Thus, the rate of exchange decreases at lower pH values (Ryle & Sanger, 1955; Eldjarn & Pihl, 1957a), and it is possible to prevent sulfhydryl/disulfide exchange by acidification (Jocelyn, 1972). Mixtures of disulfides are quite stable at pH 2.0, with no detectable interchange taking place (Spackman et al., 1960). Acidification has been used to stop sulfhydryl/disulfide exchange reactions in several kinetic studies of disulfide interchange equilibria (Eldjarn & Pihl, 1957b; Jocelyn, 1967; Gorin & Doughty, 1968). However, since dialysis and lyophilization follow the initial extraction with acetic acid, further oxidation (or exchange) is still possible. The evidence shown in Figure 2 suggests that oxidation can occur after the start of extraction of ribosomal proteins with acetic acid and before diagonal gel electrophoresis, using the standard conditions of extraction and sample preparation. The obvious difference between the "random" diagonal patterns resulting from deliberate oxidation of ribosomal proteins only after their extraction from the subunits and the pattern resulting from oxidation in the particle, indicates that the randomization reactions are slow once the disulfide bonds are formed by oxidation of the intact particle. This conclusion is substantiated by the observation that mixing of radioactive protein S4 or the dimer S5–S8 with nonradioactive cross-linked subunits did not lead to the appearance of radioactivity in any but the original species. However, it is not possible to exclude rigorously that some randomization occurs during these later steps.

Disulfide interchange and oxidation are inhibited by complete removal of free sulfhydryl groups. This may be accomplished by incubation of proteins with alkylating reagents such as *N*-ethylmaleimide and iodoacetamide (Ryle & Sanger, 1955; Creighton, 1974a,b; Lomant & Fairbanks, 1976). Iodoacetamide and iodoacetic acid have been shown by Creighton (1974a,b, 1977a) to trap intermediates in the refolding of reduced trypsin inhibitor. The same intermediates were trapped by acidification. Creighton (1975a,b, 1977b) has also provided convincing evidence that alkylation does not cause reaction of labile disulfides.

Ribosomal protein was therefore extracted in 4.0 M urea, 3.0 M LiCl (Leboy et al., 1964) as an alternative to extraction with 66% acetic acid (Hardy et al., 1969). The solution of LiCl and urea was buffered at pH 8.0 and contained a high concentration of iodoacetamide in order to block free sulfhydryl groups. Iodoacetamide was chosen as the alkylating reagent because the product of reaction is uncharged and does not change the mobility of the proteins on polyacrylamide/urea gels used in identification of the ribosomal proteins. Diagonal gel patterns of protein prepared by this method were indistinguishable from those of protein extracted with acetic acid. Because of the observation that random disulfide formation *can* take place when proteins are oxidized in acetic acid, particularly when the proteins are rich in free sulfhydryl groups, as when they have been modified with 2-iminothiolane, we conclude that the best procedure for the preparation of disulfide cross-linked proteins is extraction under denaturing conditions at pH 8.0 in the presence of an alkylating agent to block both reactive and newly exposed sulfhydryl groups. Alkylation is of particular importance when attempting to identify cross-linked proteins formed in relatively low yield.

The lack of detectable differences between diagonal patterns of protein extracted with acetic acid, or extracted at pH 8 in the presence of iodoacetamide, suggests that randomization using the former method must be slow and the products so diverse that no change in the characteristic pattern is visible. It is extremely unlikely that any cross-linked protein pairs previously identified using the acetic acid procedure (Sommer & Traut, 1974, 1975) were caused by random disulfide formation since only discrete protein spots were analyzed. Later work (Sommer & Traut, 1976) employed extraction procedures and alkylation similar to the method described here.

The yield of cross-linked dimers, as estimated by visual inspection of diagonal gel patterns, is relatively low and most likely does not exceed 20% of the total ribosomal protein. The results presented here can be used to explain this result. Modified 30S ribosomal subunits contain 61 sulfhydryl groups, of which 43 are added by modification. Between 15 and 17 free sulfhydryl groups remain even after exhaustive oxidation. If the added sulfhydryl groups were equally distributed among the 21 30S ribosomal proteins, there would be approximately two per protein. Twenty percent of cross-linked (intermolecular disulfide links) proteins would account for only about 8 of the oxidized sulfhydryl groups. The remaining 34–36 must therefore have been oxidized to form intramolecular disulfide bonds, since the results are the same when low molecular weight sulfhydryl compounds are excluded from the oxidation step. That the majority of modified lysines are so located that they readily undergo intramolecular disulfide bond formation suggests the possibility of identifying the modified regions and of determining the sites of both inter- and intramolecular cross-linking.

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