Identification of Proteins at the Subunit Interface of the *Escherichia coli* Ribosome by Cross-Linking with Dimethyl 3,3′-Dithiobis(propionimidate)†

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ABSTRACT: The 70S ribosomes of *Escherichia coli* were treated with dimethyl 3,3′-dithiobis(propionimidate). Under conditions where 40% of the lysine ϵ-amino groups became modified, about 50% of the ribosomes became resistant to dissociation into 30S and 50S subunits when analyzed in the absence of reducing agents on sucrose gradients containing low magnesium concentrations. Dissociation took place in the presence of reducing agents, indicating that the bifunctional reagent had reacted with proteins from both subunits. Proteins were extracted from purified cross-linked 70S ribosomes by using conditions to preclude disulfide interchange. Disulfide-linked protein complexes and non-cross-linked proteins were first fractionated by electrophoresis in polyacrylamide/urea gels at pH 5.5. The proteins from sequential slices of the urea gel were analyzed by two-dimensional diagonal polyacrylamide/sodium dodecyl sulfate gel electrophoresis. Monomeric proteins derived from cross-linked dimers appeared below the diagonal of non-cross-linked proteins since the second electrophoresis but not the first is run under reducing conditions to cleave the cross-linked species. Final identification of the constituent proteins in each dimer was made by radioiodination of the cross-linked proteins, followed by two-dimensional polyacrylamide/urea gel electrophoresis in the presence of nonradioactive marker 70S protein. The identification of 11 cross-linked protein dimers which contained one protein from each of the two ribosomal subunits is described. We conclude that the proteins in these cross-linked pairs are located in the regions of contact between the two subunits, i.e., at the "subunit interface".

Chemical cross-linking techniques have proven useful for determining the spatial relationships among ribosomal proteins within the ribosome. Identification of an extensive number of protein dimers permits construction of protein neighborhoods or maps which give approximate relative locations for the majority of ribosomal proteins. The use of the reversible cross-linker 2-iminothiolane and diagonal gel electrophoretic analysis of the dimers have led to the acquisition of relatively complete lists of protein neighbors in each of the purified ribosomal subunits from *Escherichia coli* (Traut et al., 1980). The reliability of the techniques used to analyze the disulfide cross-linked complexes is firmly established (Lambert et al., 1978).

A key aspect of ribosome structure and function is the existence of two subunits and their association and dissociation during the protein synthesis cycle (Davis, 1971). The association of the two ribosomal subunits involves specific structural determinates or contact regions on each subunit which can be termed the "subunit interface". Evidence from immune electron microscopy (Lake, 1980; Stöffler et al., 1980), affinity labeling (Ofengand, 1980; Cooperman, 1980), and cross-linking (Sommer & Traut, 1976; Kenny & Traut, 1979; Traut et al., 1980) suggests that the functional centers of the ribosome are located near the region of contact between the 50S and 30S subunits of the 70S ribosome. Although much of the evidence cited above implicates proteins, there is also compelling evidence showing the involvement of RNA in subunit association (Santer & Shane, 1977; Herr & Noller, 1979; Herr et al., 1979).

The present work was undertaken in order to identify proteins from both subunits in or near the subunit interface. Cross-linking of 70S ribosomes was expected to lead to the formation of hybrid dimers containing one protein from each subunit, in addition to dimers containing two proteins from the same subunit. The bifunctional imidoester dimethyl 3,3′-dithiobis(propionimidate)(DTBP)[1] (Wang and Richards, 1974) was employed to produce cross-links between proteins in 70S ribosomes. The reagent contains a disulfide bond readily cleaved by mild reduction. Identification of the cross-linked products is thus facilitated by two-dimensional diagonal polyacrylamide gel electrophoresis. The cross-linking distance of 11 Å spanned by this reagent is shorter than 14.6 Å for another reversible cross-linking agent 2-iminothiolane (Wang & Richards, 1974; Kenny et al., 1979b). A less complex diagonal pattern is formed with the shorter reagent. The results obtained with the longer reagent are the subject of a separate report (Lambert & Traut, 1981). The identification of 11 interface cross-links (dimers containing one 50S protein and one 30S protein) using DTBP is presented here. Four of these were found only with DTBP, and the others were also found by using 2-iminothiolane. The results with both reagents are discussed in relation to other types of evidence related to interface protein neighborhoods.

Materials and Methods

Materials. DTBP was purchased from Pierce Chemical Co. and stored over Drierite in a vacuum desiccator at 4 °C. Triethanolamine was from Matheson, Coleman & Bell. Acrylamide (practical grade) and *N,N′*-methylenebis(acrylamide) were from Eastman Organic Chemicals and used without further recrystallization. Urea (ultrapure) and sucrose (ribonuclease-free) were from Schwarz/Mann, 2-mercaptoethanol was from British Drug Houses, KI from Fischer Scientific, and Amido black 10B from Calbiochem. 2,4,6-Trinitrobenzenesulfonic acid, Bis-Tris, and iodoacetamide were from Sigma. Dithioerythritol, Iodo-gen, and Coomassie Blue

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[1] Abbreviations used: DTBP, dimethyl 3,3′-dithiobis(propionimidate); Iodo-gen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; mRNA, messenger RNA; tRNA, transfer RNA.

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G-250 were purchased from Pierce Chemicals. $Na^{125}I$ (carrier free) in 0.1 M NaOH was obtained from New England Nuclear.

Preparation of Ribosomes. Ribosomes were prepared from *Escherichia coli* MRE600 grown in yeast extract/glucose media (Hershey et al., 1977). In mid log phase, the cells were slow cooled to 15 °C to produce "run-off" ribosomes (Noll et al., 1973), and after 30 min at 15 °C, the cells were chilled to 0 °C and harvested. The cells were lysed by grinding with alumina in 10 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 14 mM 2-mercaptoethanol, and the ribosomes were recovered by centrifugation. The ribosomes were resuspended in the same buffer except that the $MgCl_2$ concentration was 6 mM, and 70S "tight couples" (defined as 70S ribosomes that remain associated during centrifugation in 6 mM Mg^{2+}; Hapke & Noll, 1976) were prepared by zonal centrifugation in the 6 mM $MgCl_2$ buffer with a 10%–41% (w/w) sucrose gradient (Eikenberry et al., 1970). The 70S ribosomes thus prepared were used without further purification and contained about 9% 50S subunits as judged by sucrose gradient centrifugation.

Cross-Linking of 70S Ribosomes with DTBP. Ribosomes (28 mg/mL) in 10 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, and 6 mM $MgCl_2$ were reduced by incubation with 20 mM dithioerythritol for 10 min at 37 °C. After cooling to 4 °C, the ribosomes were removed from reducing agents and amines by gel filtration through a Sephadex G-25 column (1-mL sample onto a 4.2 × 1.1 cm column) equilibrated with 50 mM triethanolamine hydrochloride, pH 8.0, 50 mM KCl, and 6.0 and mM $MgCl_2$ (TEA buffer) and diluted to 1 mg/mL with the same buffer. The ribosomes were incubated in 20 mM DTBP for 4 h at 0 °C. Stock solutions (0.5 M) of DTBP were made by dissolving the dry solid in a solvent consisting of equal volumes of 2.0 M triethanolamine free base and 1.0 M triethanolamine hydrochloride, pH 8.0; the final pH was about 8.0, and the solution was used within 1 min.

The extent of modification of ribosomes by DTBP was estimated by titration of free amino groups with trinitrobenzenesulfonic acid as described previously (Lambert & Perham, 1977). The effect of cross-linking on the activity of the ribosomes in poly(U)-dependent polyphenylalanine synthesis was assayed by the method of Lacoste et al. (1976). The sedimentation properties of the cross-linked particles were examined by sucrose gradients (7% to 25% w/w) prepared in 10 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, and either 10 mM or 1.1 mM $MgCl_2$ in the presence or absence of reducing agents (20 mM 2-mercaptoethanol). Samples were centrifuged at 16 500 rpm for 16.5 h at 4 °C with a Beckman SW56 rotor.

Extraction of Proteins from Cross-Linked Ribosomes. Following cross-linking with DTBP, 70S ribosomes were purified from excess reagents, contaminating 50S subunits, and from a small amount (about 12%) of material sedimenting at about 100 S, which probably represents 70S dimers, by centrifugation in sucrose gradients (7% to 25%, w/w) in 10 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl and 10 mM $MgCl_2$ by using a Beckman SW27 rotor at 17 000 rpm for 12.5 h at 4 °C. Ribosomal proteins were extracted from the cross-linked 70S particles in the presence of 100 mM iodoacetamide as a precaution to prevent disulfide interchange catalyzed by free sulfhydryl groups (Lambert et al., 1978; Lambert & Traut, 1981).

Fractionation of Proteins Extracted from Cross-Linked Ribosomes by Polyacrylamide/Urea Gel Electrophoresis. Lyophilized proteins (7.0 mg extracted from about 21.0 mg of purified, cross-linked 70S ribosomes) were dissolved in 8.0 M urea, 20 mM Bis-Tris–acetate, pH 3.7, and 40 mM iodoacetamide (150 μL). Electrophoresis in a gel tube (13 × 0.6 cm) of 4% (w/v) acrylamide, 0.066% (w/v) bis(acrylamide), 38 mM Bis-Tris, 6.0 M urea, and 1.0 mM iodoacetamide adjusted to pH 5.5 with acetic acid, with an upper gel (0.5 × 0.6 cm) of the same composition adjusted to pH 4.7, was performed as described previously (Lambert & Traut, 1981; Kenny & Traut, 1979). After electrophoresis, the gel was sliced into 27 equal fractions 0.5 cm in length.

Analysis of Urea Gel Fractions by Two-Dimensional Diagonal Polyacrylamide/Sodium Dodecyl Sulfate Gel Electrophoresis. The analysis of protein dimers contained in each 0.5-cm urea gel fraction was made by diagonal gel electrophoresis, which exploits the fact that the disulfide bond linking two proteins can be cleaved by reduction to regenerate the monomeric protein components. The details of the procedures used to analyze the urea gel fractions were exactly as described by Lambert & Traut (1981), and a detailed description of two-dimensional diagonal polyacrylamide/sodium dodecyl sulfate gel electrophoresis can be found elsewhere (Kenny et al., 1979b; Kenny & Traut, 1979; Sommer & Traut, 1976). Calibration of the mobility of the proteins with respect to molecular weight was made by using molecular weights for the ribosomal proteins calculated from their amino acid sequences [summarized by Wittmann et al. (1980); Brimacombe et al. (1978)]. The positions for most of the monomeric proteins are shown in Figure 3a. The coincidence of protein spots on a vertical axis below the diagonal provides one of two major criteria for identifying pairs of proteins originally cross-linked in the intact ribosome. The other criterion is that the sum of the molecular weights of the monomeric proteins in a putative dimer must be approximately the same as the apparent molecular weight of the cross-linked complex estimated by its relative mobility in the first dimension of electrophoresis.

Identification of Proteins Contained in Protein Spots Isolated from Diagonal Gels. The position of a protein spot below the diagonal often does not permit unambiguous identification because many of the ribosomal proteins have similar molecular weights and similar mobilities on polyacrylamide/sodium dodecyl sulfate gels. Therefore, the proteins contained in the protein spots below the diagonal were extracted from the gel and analyzed further by a radioiodination procedure described in detail by Tolan et al. (1980). Addition of 0.05 mM EDTA and 0.1 mM dithioerythritol to the sodium dodecyl sulfate extraction buffer which had been deaerated and saturated with nitrogen resulted in improved recovery of protein from the gel. Proteins L20 and S12 comigrate in diagonal gel electrophoresis and in the two-dimensional polyacrylamide/urea gels used to identify the radioiodinated proteins. These proteins were again eluted from the gel and analyzed in a third polyacrylamide gel system containing urea and sodium dodecyl sulfate (Mets & Bogorad, 1974) as described by Lambert & Traut (1981).

Results

Cross-Linking of 70S Ribosomes with DTBP. Figure 1a,b shows that the sedimentation properties of the 70S ribosomes were unchanged by modification with 20 mM DTBP when analyzed in sucrose gradients containing 10 mM Mg^{2+}. Figure 1c shows that a substantial proportion (abut 45%) of the modified ribosomes was resistant to dissociation when analyzed in sucrose gradients containing 1.1 mM Mg^{2+} in the absence of reducing agents. In the presence of reducing agents (Figure 1d), the majority of the modified ribosomes sedimented as 50S and 30S subunits. The small shoulder sedimenting at greater

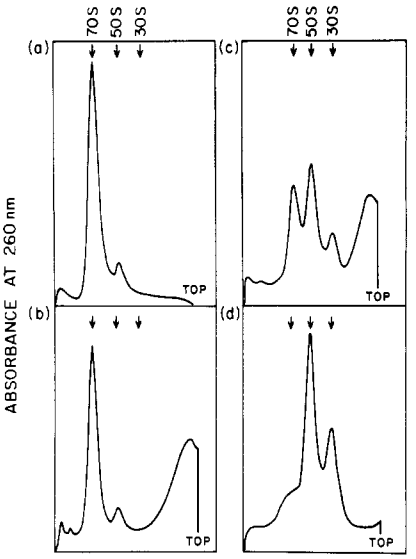

FIGURE 1: Analysis of cross-linked 70S ribosomes by sucrose gradient centrifugation. Samples of ribosomes (60 μg) were analyzed by using conditions described under Materials and Methods. (a) Unmodified (control) ribosomes analyzed in a gradient containing 10 mM $MgCl_2$ buffer without reducing agents. (b) Ribosomes cross-linked with DTBP (20 mM) and analyzed under the same conditions as gradient a. (c) Identical with (b) except that the gradient buffer contained only 1.1 mM $MgCl_2$. (d) Identical with (c) except that the cross-linked ribosomes were reduced before centrifugation and run in gradients containing 20 mM 2-mercaptoethanol. The high absorbance at the top of gradients b and c was due to DTBP.

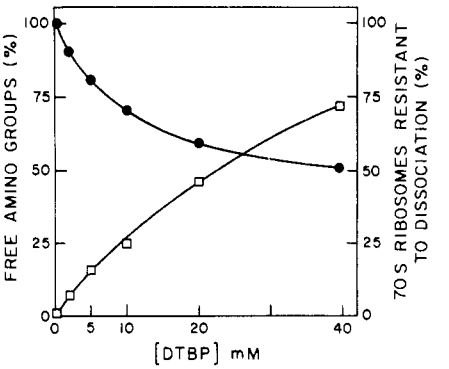

FIGURE 2: The effect of DTBP concentration on the number of free ribosomal amino groups and on the resistance of ribosomes to subunit dissociation induced by low Mg^{2+} concentrations. Free amino groups were determined by assay with trinitrobenzenesulfonic acid, and the resistance of 70S ribosomes to dissociation was quantified in sucrose gradients under the same conditions as in Figure 1c. For further details, see Materials and Methods. (●) Free amino groups; (□) the proportion of 70S ribosomes that does not dissociate in sucrose gradients containing 1.1 mM $MgCl_2$ in the absence of reducing agents.

than 50S (Figure 1d) was likely due to incomplete reduction or to reoxidation during centrifugation. Such material was not seen after modification of 70S ribosomes with the monofunctional reagent methyl acetimidate, suggesting that it was not produced by the formation of noncleavable cross-links which have been observed occasionally during modification with imidoesters (Browne & Kent, 1975).

Figure 2 shows the effect of DTBP concentration on the number of ribosomal amino groups under the standard conditions of modification described under Materials and Methods. The proportion of the 70S ribosomes resistant to dissociation, determined by sucrose gradient centrifugation under conditions identical with those for Figure 1c, is also shown in Figure 2. For our standard conditions of cross-linking, we chose the DTBP concentration (20 mM) which rendered about 50% of the ribosomes resistant to dissociation and led to the amidination of about 40% of the total amino groups.

The effect of modification on poly(U)-dependent polyphenylalanine synthesis was also tested. Particles cross-linked with 20 mM DTBP showed only 30–40% of the activity of control ribosomes when assayed in the absence of reducing agents. However, when assayed under reducing conditions after prior reduction of the modified particles, the ribosomes had 90% of the activity of controls.

Analysis of Proteins from Cross-Linked 70S Ribosomes by Diagonal Gel Electrophoresis. Figure 3a shows a diagonal polyacrylamide/sodium dodecyl sulfate gel of monomeric proteins extracted from non-cross-linked 70S ribosomes. The position of each monomeric protein as it falls on the diagonal is designated by a letter shown at the right of Figure 3a; also shown are the (average) molecular weight(s) of the protein(s) on each lettered row, calculated from their primary structures as described in the legend to Figure 3.

The diagonal gel system shown in Figure 3a was adequate to analyze the proteins contained in each urea gel fraction of cross-linked ribosomal proteins (Figure 4). However, it proved difficult to obtain a clear pattern of cross-linked total protein from 70S ribosomes on such diagonal gels. It was likely that the poor resolution was associated with the high protein loading necessary and with the high degree of amino group modification (Figure 2). These conditions would favor reoxidation of sulfhydryl groups in the second dimension of diagonal electrophoresis. For improvement of resolution, an acrylamide gradient gel was used in each dimension, and the first dimension gel cylinder was embedded at the top of the second dimension gel with 1% (w/v) agarose containing 10% (v/v) 2-mercaptoethanol. Figure 3b shows such a modified diagonal gel of a sample of proteins extracted from cross-linked 70S ribosomes in the presence of high concentrations of iodoacetamide to preclude disulfide interchange catalyzed by sulfhydryl groups (Lambert et al., 1978). Monomeric proteins derived from disulfide cross-linked complexes can be seen below the "diagonal" line of monomeric proteins, showing that DTBP had cross-linked lysine residues between many different ribosomal proteins.

Fractionation of Cross-Linked Proteins and Analysis of Fractions by Diagonal Gel Electrophoresis. The diagonal pattern resulting from total cross-linked protein from 70S ribosomes is necessarily quite complex (Figure 3b). Interpretation of the complex pattern, matching of monomeric proteins arising from cross-linked dimers, is made difficult due to the fact that many monomeric proteins and many cross-linked species have coincident or quite similar mobilities. To overcome these difficulties, the mixture of cross-linked proteins was fractionated prior to diagonal gel analysis. Fractionation was achieved by polyacrylamide/urea gel electrophoresis as described under Materials and Methods; the proteins predominantly separate in this system according to their net charge. The proteins in each fraction were then analyzed by diagonal sodium dodecyl sulfate gel electrophoresis, using the gel system characterized in Figure 3a. A gallery of the diagonal gels of the first 20 fractions obtained by electrophoretic fractionation is shown in Figure 4. The remaining seven fractions not shown (21–27) contained only low molecular weight monomeric proteins. The fractionation greatly simplified the patterns of protein spots below the diagonals with an accompanying enrichment of certain protein pairs in each diagonal gel.

Several simplified diagonal gels are shown in Figures 5–7 in order to illustrate each of the cross-linked protein pairs described in this paper. Protein spots below the diagonals are designated according to the lettering scheme shown in Figure

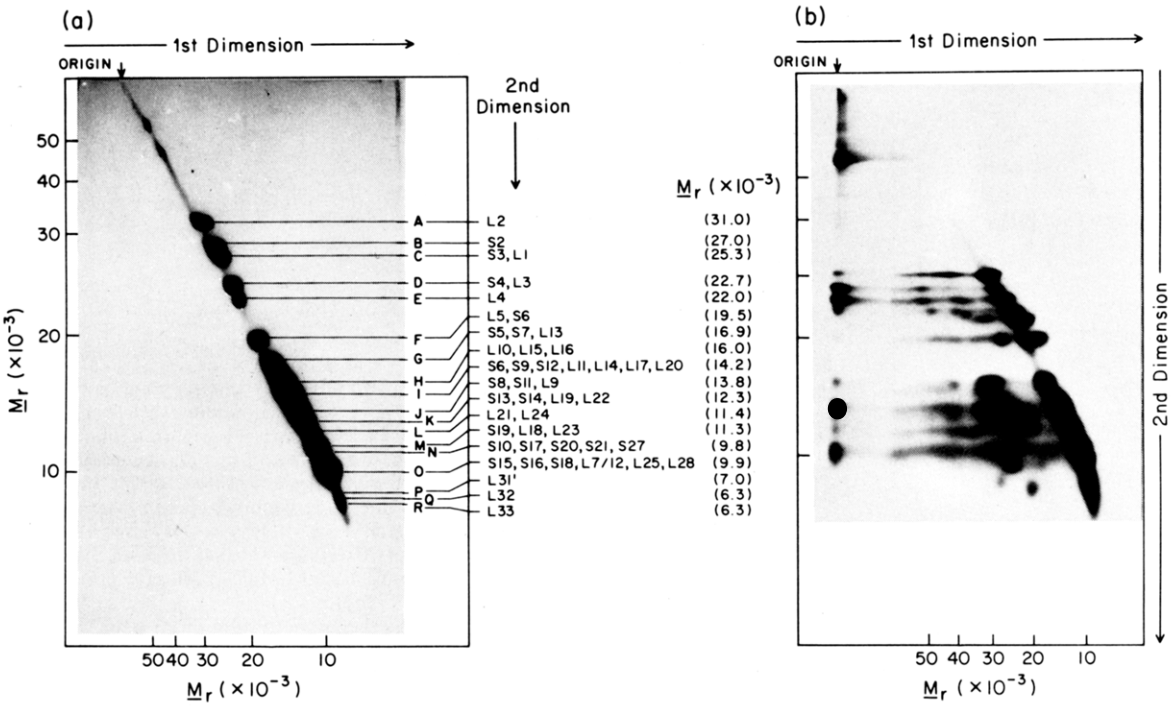

FIGURE 3: Two-dimensional diagonal polyacrylamide/sodium dodecyl sulfate gel electrophoresis of proteins extracted from 70S ribosomes. (a) Proteins (800 μg) from unmodified 70S ribosomes. The first dimension was 14.5% (w/v) acrylamide and 0.38% (w/v) bis(acrylamide), and the second dimension was 17.5% (w/v) acrylamide and 0.35% (w/v) bis(acrylamide). This gel system was used for all diagonal gels shown in Figures 4–7. The techniques have been described in detail and are referred to under Materials and Methods. The position of each 70S protein on the diagonal line of monomeric proteins was determined as described previously (Kenny & Traut, 1979; Lambert & Traut, 1980). The molecular weight (M_r) calibration for both dimensions was established by using molecular weights for the ribosomal proteins calculated from amino acid sequence evidence (Wittmann et al., 1980). The molecular weights used for proteins which have not yet been sequenced, L2 (31 000), S2 (27 000), L4 (22 000), L9 (13 700), and L17 (14 000), were determined from the curves of log molecular weight vs. electrophoretic mobility plotted for all the other ribosomal proteins. The average M_r of the proteins on each of the lettered rows are shown at the right of panel a. Positions are shown for all the ribosomal proteins except L29, L30 and L34. (b) Proteins (1.0 mg) extracted from 70S ribosomes cross-linked with 20 mM DTBP and then purified by sucrose gradient centrifugation as described under Materials and Methods. The first and second dimensions of this gel were cast with acrylamide gradients of 8%–19% (w/v) and 12.5%–20% (w/v), respectively. The acrylamide/bis(acrylamide) ratio was 30:0.8. The first dimension gel cylinder was reduced with 5% (v/v) 2-mercaptoethanol and embedded at the top of the second dimension gel slab with 1% (w/v) agarose containing 10% (v/v) 2-mercaptoethanol. The monomeric components of a disulfide cross-linked complex migrate to positions below the diagonal line of non-cross-linked proteins in the second dimension. For other details, see the description for panel a and the text.

3a which indicates the horizontal rows which contain all proteins of the same mobility in the second dimension. Members of a cross-linked complex are matched by their coincidence on a single vertical axis and the additivity of their molecular weights to give that of the parent dimer. Each vertical axis in Figures 5–7 is labeled with the apparent molecular weight $\times 10^{-3}$ of the cross-linked complex. A cross-linked pair is thus identified by one number (apparent $M_r \times 10^{-3}$) and two letters (one for each protein spot).

Identification of Cross-Links Involving Proteins from both 30S and 50S Subunits: An Illustrative Example Describing the Analysis of Protein Pairs from Urea Gel Fraction 16. Figure 5a shows the simplified diagonal gel pattern from urea gel slice 16. The vertical line designated 29.5 intercepts two conspicuous spots on rows G and I (refer to Figure 3a for the proteins contained in each lettered row). Proteins were eluted from the spots and radioiodinated as described under Materials and Methods. Figure 5b shows the radioautograph of the two-dimensional polyacrylamide/urea gel of the radioiodinated protein from spot G. The radioactive species coincides with protein L13. The dotted outlines indicate the positions of stained material representing the nonradioactive carrier protein added as reference. The radioautograph shown in Figure 5c identified the protein from spot I as either S12 or L20 or both proteins. To discriminate between S12 and L20, the radioiodinated protein was again eluted from the gel and analyzed in another electrophoretic system (Figure 5d). The radioautograph shown in lane 4 (figure 5d) identified the protein as S12. The sum of the molecular weights (S12 + L13) is 29 600 (Wittmann et al., 1980), in good agreement with the apparent molecular weight of the parent cross-linked species (Figure 5a). We thus conclude that pair 29.5 (spots G and I) was due to the cross-link S12–L13.

The spots on rows K and M (Figure 5a), lying on the vertical 25.4, were analyzed similarly. Spot 25.4 K contained S13 while spot 25.4 M was found to be L18. Thus these spots were derived from an S13–L18 cross-linked pair.

Identification of Cross-Links Involving Proteins from both 30S and 50S Subunits in Urea Gel Fractions 9 and 11. Figure 6a shows the diagonal pattern of urea gel slice 9. Two protein pairs are indicated by the vertical axes 52.0 and 39.3. Radioautographs identified spot 52.0 B as S2 while spot 52.0 C contained both L1 and S3. Thus pair 52.0 was derived from two cross-links, S2–S3 (Sommer & Traut, 1976) and S2–L1. The spots on rows C and J of pair 39.3 were identified as proteins L1 and S8, respectively, showing a cross-link S8–L1.

Two protein pairs are indicated in Figure 6b, which shows the diagonal gel of urea gel fraction 11. Spot D of pair 35.7 was found to contain both S4 and L3, while spot K (35.7) contained only L19. We conclude that pair 35.7 was derived from the cross-links S4–L19 and L3–L19. Pair 23.3 matched spots on rows J and O. Spot J was found to contain L9 and S8 while spot O contained L28. Thus pair 23.3 was also derived from two cross-links, L9–L28 and S8–L28.

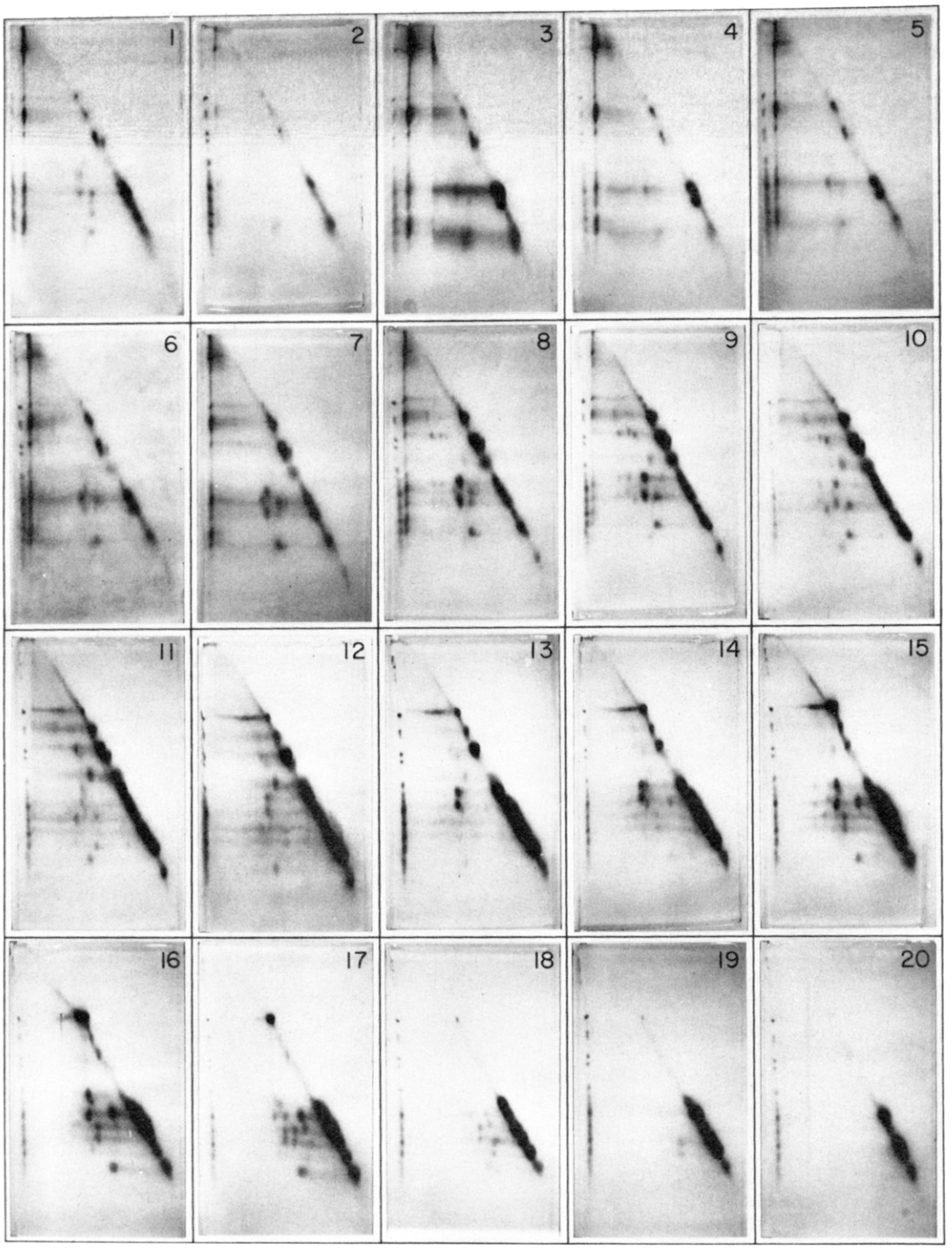

FIGURE 4: A gallery of diagonal gels of fractionated proteins extracted from cross-linked 70S ribosomes. The mixture of ribosomal proteins (7.0 mg) was fractionated by polyacrylamide/urea gel electrophoresis in a gel of 4% (w/v) acrylamide, pH 5.5. For further details, see Materials and Methods. Each 0.5-cm slice of the urea gel (27 slices) was analyzed by diagonal gel electrophoresis. The figure shows the diagonal gels for the first 20 sequential fractions.

Identification of Cross-Links Involving Proteins from both 30S and 50S Subunits in Urea Gel Fractions 17 and 18. Figure 7a illustrates the separation of proteins in urea gel slice 17 by diagonal gel electrophoresis. One pair is indicated on the vertical axis labeled 20.4. The spot on row I contained L17, S9, and S12 while the spot on row Q was identified as L32. The presence of the three proteins in the same spot must be due to dimer formation between each one and L32. Dimers containing two I species would have an apparent molecular weight greater than 20400. Thus cross-link 20.4 (I,Q) consists of three dimers, L17–L32, S9–L32, and S12–L32.

Figure 7b shows the diagonal gel of urea gel fraction 18. Three pairs are indicated. Pair 29.3 matched spots on rows H and I. Spot 29.3 H was identified as L16 while spot 29.3 I was S12. The evidence shows that S12 cross-links to L16. Another spot on row I was matched with a spot on row L by the vertical axis 25.4. Spot 25.4 I contained S12 while spot 25.4 L contained L21, showing the pair was due to the cross-link S12–L21. The spot on row N of pair 15.5 was found to contain S21 and L23 while spot 15.5 R contained only L33. We conclude that pair 15.5 was derived from two cross-links, S21–L33 and L23–L33.

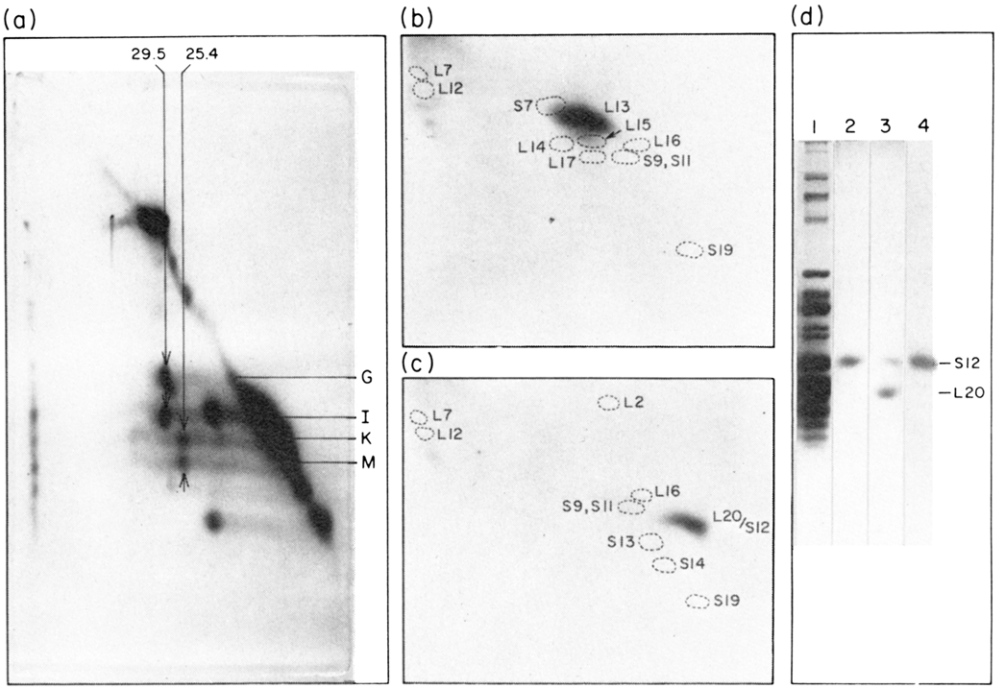

FIGURE 5: Identification of cross-links containing proteins from each of the 30S and 50S subunits in urea gel fraction 16. (a) Diagonal gel pattern of proteins from cross-linked 70S ribosomes in urea gel fraction 16. Refer to Materials and Methods for details of protein extraction and fractionation. Proteins that were cross-linked in the first dimension fall below the diagonal line in the second dimension. Vertical lines, with arrows, indicate two of these protein pairs. The vertical axes are numbered 29.5 and 25.4, a number which equals the apparent molecular weight $\times 10^{-3}$ of the cross-linked pair in the first dimension. The molecular weight calibration was as described under Materials and Methods. The spots shown by the arrows on each vertical axis are assigned to horizontal lettered rows, with the letters corresponding to the letter code shown in Figure 3a. Thus the spot characterized by vertical 29.5 and letter G could be one (or two, or all) of the proteins S5, S7, and L13. Correlation of the spots, both on and beneath the diagonal, to the gel pattern of Figure 3a was facilitated by the electrophoresis of unmodified total 50S protein to form the column of marker proteins on the left of the diagonal gel: total 50S proteins (50 μg in 10 μL of sample buffer) were applied to the top of the first dimension gel tube 10 min before the end of electrophoresis. (b) Radioautograph of a two-dimensional polyacrylamide/urea gel of radioiodinated protein(s) from spot 29.5 G. For details of the radioiodination procedure, see Materials and Methods and references therein. The nonradioactive carrier/marker proteins (100 μg of total 70S protein) were stained with Coomassie Blue G-250; stained protein spots in the vicinity of the radioactive proteins are indicated by broken circles drawn on the radioautography [for a complete pattern of ribosomal proteins in this gel system, refer to Tolan et al. (1980) or Kenny et al. (1979a,b)]. The radioautograph was superimposed on the stained gel to identify the radioactive protein spots. (c) Radioautography of a two-dimensional polyacrylamide/urea gel of radioiodinated protein from the spot 29.5 I. (d) One-dimensional polyacrylamide/sodium dodecyl sulfate/urea gel electrophoresis of ^{125}I-labeled proteins extracted from the unresolved radiolabeled L20/S12 spot of the gel that gave the radioautograph shown in (c). (Lane 1) Total 50S protein (40 μg), used as carrier and marker). (Lane 2) Pure S12 from total 30S protein. (Lane 3) A mixture of S12 and L20 from total 70S protein. Lanes 1–3 were stained with Coomassie brilliant blue. The samples of protein for lanes 2 and 3 were prepared by using procedures similar to those of Cleveland et al. (1977). Pieces of gel containing S12 or S12/L20 were excised from two-dimensional polyacrylamide/urea gels of total 30S protein or total 70S protein, respectively. The gel pieces were soaked in sample buffer containing sodium dodecyl sulfate, inserted into wells of the gel slab, and submitted to electrophoresis (Mets & Bogorad, 1974). Lane 4 shows the radioautograph of radiolabeled protein from the S12/L20 spot from (c).

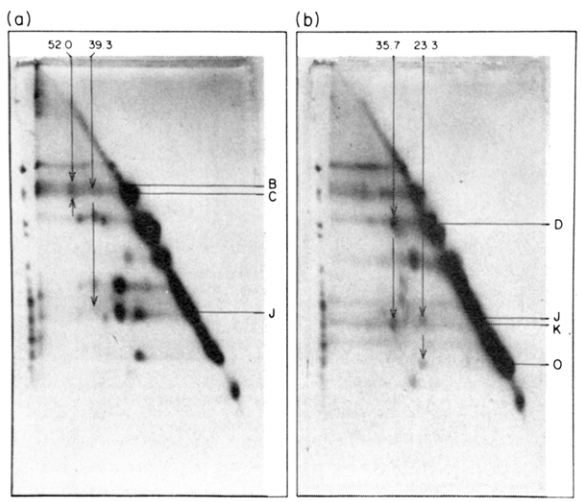

FIGURE 6: Identification of cross-links containing proteins from each of the 30S and 50S subunits in urea gel fractions 9 and 11. (a) Diagonal gel pattern of proteins from cross-linked 70S ribosomes in urea gel fraction 9. (b) Diagonal gel pattern of proteins in urea gel fraction 11. For further details, see the legend to Figure 5 and the text.

Summary of Cross-Linked Protein Dimers Each Involving One Protein from Each of the 30S and 50S Subunits ("*Interface Cross-Links*"). Table I summarizes the molecular weight and final identification of 11 hybrid protein pairs, or "interface cross-links", that result from a direct cross-link between one protein from the 30S subunit and a second protein from the 50S subunit. The apparent molecular weights of the cross-links obtained with DTBP agree very closely with the molecular weight for each cross-link calculated from the sum of their monomeric molecular weights (Table I). The 11 interface cross-links were identified following the characterization of all protein spots beneath all the diagonals shown in Figure 4; additional interface cross-links, if present at all, were formed in such low yields as to be undetectable in these experiments. Many of the cross-linked complexes involved only 30S proteins or only 50S proteins; some of these were mentioned above while the procedures for identifying all the interface cross-links were being described. A complete account of the dimers within each subunit formed by cross-linking 70S ribosomes will be presented elsewhere (J. M. Lambert, G. Boileau, J. W. Kenny, and R. R. Traut, unpublished results).

It is difficult to assess precisely the relative yields of the

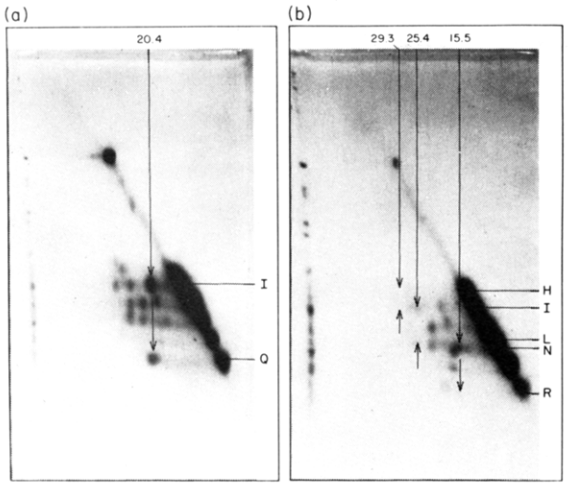

FIGURE 7: Identification of cross-links containing proteins from each of the 30S and 50S subunits in urea gel fractions 17 and 18. Fraction 17 (a) and fraction 18 (b) were analyzed by diagonal gel electrophoresis. For further details, see the legend to Figure 5 and the text.

Table I: Characterization and Identification of Cross-Links Containing One Protein from Each of the 30S and 50S Subunits

cross-link	urea gel fraction[a]	apparent M_r of cross-linked complex[b] $\times 10^{-3}$	sum of M_r of monomeric proteins[c] $\times 10^{-3}$	relative yield (H, I, L)[d]
S2–L1	9	52.0	51.6	I
S4–L19	11	35.7	35.1	I
S8–L1	9	39.3	38.6	I
S8–L28	11	23.3	22.9	I
S9–L32	17	20.4	20.9	L
S12–L13	16	29.5	29.6	H
S12–L16	18	29.3	28.9	L
S12–L21	18	25.4	25.2	L
S12–L32	17	20.4	19.9	I
S13–L18	16	25.4	25.8	I
S21–L33	18	15.5	14.7	L

[a] Dimers were identified in the diagonal gels of several urea gel fractions (see Figure 4). The gel used to illustrate each cross-link in Figures 5–7 is listed here. [b] Determined from the relative mobility in the first dimension of diagonal electrophoresis, under oxidizing conditions. [c] The molecular weight values used for the monomeric proteins were calculated from their primary sequences (Wittmann et al., 1980). [d] The relative yield is a qualitative estimate based on a visual inspection of the relative intensity of spots below the diagonal and on the number of successive fractions where a particular pair is visible. Three categories are defined as follows: H (higher), among the strongest spots below the diagonal, appearing in about 3–5 successive fractions; I (intermediate), spots below the diagonal of average intensity, visible in 2–3 successive diagonals; L (lower), among the weakest spots below the diagonal and found in only 1–2 successive diagonal gels.

different dimers. Rough estimates were possible from the staining intensities of the protein spots and from the number of successive gel slices (see Figure 4) in which cross-links appear. On this basis, cross-links S9–L32, S12–L16, S12–L21, and S21–L33 were judged to have been formed in lower yields than the other seven cross-links (Table I), while the cross-link S12–L13 was the interface cross-link found in highest yield with DTBP.

Discussion

The protein neighborhoods of the *Escherichia coli* 70S ribosome were investigated by using the bifunctional reversible cross-linking reagent DTBP. The ribosomes were "tight couples" presumed to lack peptidyl-tRNA and mRNA and were assumed to retain the general features of the interface of functioning particles. This paper reports the identification of 11 hybrid cross-linked pairs of proteins that contain one protein from each subunit (Table I). We conclude that the proteins found in hybrid dimers are at the interface between the small and the large subunits of the 70S ribosome and refer to the cross-linked dimers as "interface cross-links". Four of the interface cross-links reported here, S4-L19, S8–L1, S12–L13, and S21–L33, were not identified in a study with the longer reagent 2-iminothiolane (Lambert & Traut, 1981); the remaining seven interface cross-links were found with both reagents. Table II lists all 27 interface cross-links identified in 70S ribosomes with DTBP reported here and with 2-iminothiolane (Lambert & Traut, 1981). A separate study showed that protein S9 was present on 50S subunits and was cross-linked to L5 and L27 (Kenny et al, 1979a). These cross-links are also listed in Table II.

Table II: Summary of Interface Cross-links (30S Proteins Listed First)

S2–L1[a,b]	S12–L2[a]
S4–L1[a]	S12–L13[b]
S4–L17[a]	S12–L16[a,b]
S4–L19[b]	S12–L21[a,b]
S6–L10[a]	S12–L32[a,b]
S8–L1[b]	S13–L5[a]
S8–L28[a,b]	S13–L6[a]
S9–L1[a]	S13–L18[a,b]
S9–L2[a]	S13–L19[a]
S9–L5[c]	S15–L2[a]
S9–L27[c]	S19–L5[a]
S9–L32[a,b]	S19–L16[a]
S10–L1[a]	S19–L32[a]
S11–L1[a]	S21–L33[b]
S11–L2[a]	

[a] Lambert & Traut (1981). [b] This paper. [c] Kenny et al. (1979a).

Twelve 30S proteins and fifteen 50S proteins form hybrid dimers at the subunit interface when 70S ribosomes are treated with the disulfide cross-linking reagents (Table II). About half of all the ribosomal proteins have at least part of their structures within the interface, i.e., near enought to the other subunit to be accessible to the cross-linking reagents. When the reagent DTBP, for which the maximum distance between cross-linked residues is 3.6 Å less than that for 2-iminothiolane, is used, fewer interface dimers, 11 vs. 23, were formed as compared to the longer reagent. The results suggest that the extra length afforded with the longer reagent may, in certain cases, facilitate the formation of cross-links between proteins less intimately involved in the interface. However, it is difficult to ascertain the role played by differing reactivities of the two reagents. One of the interface proteins identified with DTBP, protein L13, was not found with 2–iminothiolane in any of the more numerous hybrid cross-links produced by that reagent. Cross-links containing proteins L33 and S21 could only be partially characterized in the experiments with 2-iminothiolane (Lambert & Traut, 1981). Other members of the cross-linked pairs *unique* to DTBP (S4, L19, S8, L1, and S12) were nevertheless defined as interface proteins by their presence in other 2-iminothiolane hybrid cross-links.

The mechanism of protein synthesis entails the reversible association of the two ribosomal subunits (Davis, 1971). The contact region or interface between them is of special functional importance since the results of many experiments directed at the identification of functional sites imply the location of these active sites in the interface neighborhood. There is an excellent correlation between the interface proteins defined directly by cross-linking and those implied by other types of experiments.

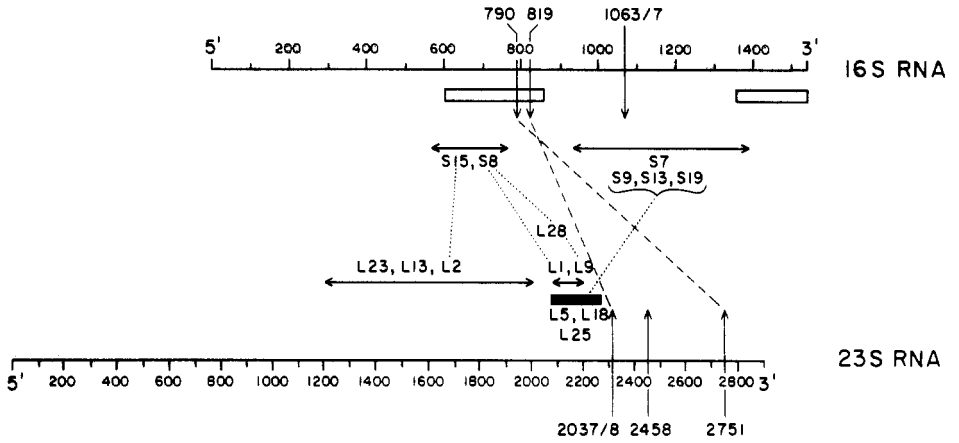

FIGURE 8: Schematic diagram of RNA sites and some proteins involved in the ribosomal subunit interface. The scales denote nucleotide distance from the 5′ termini of the RNA molecules. The open bars indicate regions of the 16S RNA containing many guanine residues essential for subunit association (Herr et al., 1979). The vertical arrows indicate particular guanine residues essential for subunit association mentioned in the text; they are numbered according to the RNA sequences determined by Brosius et al. (1978, 1980). The horizontal arrows show the approximate locations of specific ribosomal protein binding site fragments (Müller et al., 1979; Zimmermann, 1974; Branlant et al., 1976; Spierer et al., 1979). The solid bar indicates the approximate location of the binding site of the 5S RNA complex containing proteins L5, L18, and L25 (Branlant et al., 1976). The thin dashed lines connect RNA sites that have potential for base-pair interactions (Herr et al., 1979; Brosius et al., 1980). The dotted lines connect proteins involved in interface cross-links (Table II).

The protection of tRNA from nuclease digestion when it is bound to 70S ribosomes (Kuechler et al., 1972) suggests that it binds at the interface region. Proteins affinity labeled by tRNA derivatives are very likely located in the interface neighborhood. The cross-linked interface proteins L2, L16, L18, L27, and S4 are among a much larger group affinity labeled by aminoacyl derivatives of tRNA or by puromycin and derivatives thereof [summarized in Ofengand (1980); Cooperman, 1980]. Protein S19, another cross-linked interface protein, is affinity labeled by a probe attached to the central region of tRNA (Ofengand, 1980). Protein L16 is an interface protein from the cross-linking experiments. It is also an important component of the peptidyl transferase center (Baxter & Zahid, 1978; Moore et al., 1975; Teraoka & Nierhaus, 1978). The evidence indicates that the location of this functional site is at the interface, the region of the 50S subunit close to or in contact with the 30S subunit. Interface cross-links involving proteins L5 and L18 imply that 5S RNA, together with the proteins that bind to it, occupies a site in the interface neighborhood. It has been suggested previously that 5S RNA acts as a bridge between ribosomal subunits (Azad & Lane, 1973).

A role in interactions involving mRNA has been indicated for several of the interface proteins identified by cross-linking. Proteins S4 and S12, found in interface cross-links, are among a group of proteins labeled by mRNA affinity probes (summarized in Cooperman, 1980). Protein S11 is also found in interface cross-links while the occurrence of S5 in both subunits suggests that it is an interface protein (Kenny et al., 1979a). All four of these proteins have effects on the error frequency of translation (Gorini, 1971; Nomura et al., 1969; Stöffler et al., 1971).

Proteins S1, S11, S12, S13, and S19 have been directly or indirectly cross-linked to two or three of the initiation factors (Bollen et al., 1975; Heimark et al., 1976b; Langberg et al., 1977). All of these proteins except S1 (G. Boileau, and R. R. Traut, unpublished results) are found among the interface cross-links. This indicates that the initiation site of the small subunit faces toward the large subunit.

The interaction of the ribosome with elongation factors G and Tu also involves sites near the interface. Both factors have been cross-linked to protein L1 (Maassen, 1979; San José et al., 1976), a protein found in six interface cross-links at least one of which, L1–S2, is formed in high yield (Lambert & Traut, 1981). Elongation factor G and initiation factor 2 have also been cross-linked to L7/L12 (Acharya et al., 1973; Heimark et al., 1976a), proteins thought to comprise a conspicuous asymmetric feature of the large subunit (Boublik et al., 1976; Strycharz et al., 1978). That L7 and L12 are not found in interface cross-links suggests that they are not directly involved in subunit interaction or in direct contact with the 30S subunit. In summary, comparison of the proteins inferred to be at the interface from the results of cross-linking presented here with those for which functional roles have been attributed from other experimental approaches leads to the conclusion that the interface neighborhood of each subunit contains, at least in part, proteins comprising ribosomal functional centers.

Ribosomal RNA plays an important functional role (Shine & Dalgarno, 1974; Steitz & Jakes, 1975; Breitmeyer & Noller, 1976; Zimmermann et al., 1979), and the RNA probably plays a role in determining and stabilizing the interactions between the subunits. Figure 8 is a scheme which shows the correlation between interface protein cross-links and evidence concerning the involvement of RNA at the interface. Results of modification experiments with kethoxal, correlating loss of capacity of 30S subunits to associate with 50S subunits with modification of guanine residues, showed that sites essential for subunit association in 16S RNA were clustered between residues 600 and 850 and between 1350 and 1541 as shown in Figure 8 (Herr et al., 1979). The 3'-terminal region (residues 1350–1541) has also been implicated in initiation of protein synthesis (Shine & Dalgarno, 1974; Steitz & Jakes, 1975). The region from residues 850 to 1350 contained only one residue essential for subunit association (Herr et al., 1979), shown by the arrow labeled 1063/7 in Figure 8. In addition, several residues in this region of the 16S RNA were partially protected from kethoxal when 30S subunits were associated with 50S subunits while some sites became more reactive (Herr et al., 1979). Similar experiments with 50S subunits showed only two guanine sites essential for association (Herr & Noller, 1979), at residues 2307/8 and 2458 in the 3′-third of the 23S RNA molecule (Brosius et al., 1980). The positions of these sites, and a site in the 23S RNA that was partially protected by association with 30S subunits, are shown by arrows in Figure 8. The nucleotide sequences near the partially protected residue 2751 and around residue 2308 have the potential for

forming base-pair interactions with sequences around residues 790 and 817 in the middle region of the 16S RNA (Herr et al., 1979; Brosius et al., 1980). These potential interactions are represented by dashed lines in Figure 8.

The RNA binding sites for many ribosomal proteins have been established. Many of the interface proteins as inferred from cross-linking bind to the regions of the RNA thought to be related to the interface as summarized above. Proteins S8 and S15 bind strongly to the 16S RNA between residues 570 and 760 (Müller et al., 1979), and it appears that the secondary structure of this RNA site is conserved in evolution (Stanley & Ebel, 1977). This region is mostly within the interface domain, residues 600–850 (Figure 8), described on the basis of the kethoxal modification studies (Herr et al., 1979). Protein S8 forms the interface cross-links S8–L1 (this work) and S8–L28 (Table II), while S15 forms the cross-link S15–L2 (Lambert & Traut, 1981). Protein L1 has a discrete binding site on the 23S RNA between residues 2087 and 2201 (Branlant et al., 1976; Brosius et al., 1980), and the RNA secondary structure of this site seems to be highly conserved (Stanley et al., 1978). Figure 8 shows that the L1 site, like the S8 site, is adjacent to RNA sites found essential for subunit association. In Figure 8, the cross-links S8–L1 and S15–L2 are represented by dotted lines. The relationship between the S8 binding region on the 16S RNA and the L1 binding region on the 23S RNA is further strengthened by the cross-links S8–L28 and L9–L28, found in good yield with both 2-iminothiolane (Lambert & Traut, 1981) and DTBP. Protein L9 binds to the L1 RNA site (Branlant et al., 1976), and so L28 can be viewed to bridge the S8 and the L1/L9 RNA binding sites (Figure 8).

The complex of 5S RNA and the proteins L5, L18, and L25 binds to the 23S RNA in the region of the L1/L9 binding site (Branlant et al., 1976). The interface cross-links L5–S9, L5–S13, L5–S19, and L18–S13 strongly relate the 5S RNA complex to 30S proteins that associate with the region of the 16S RNA between residues 850 and 1350 (Zimmermann, 1974). The 5S site and the tRNA site appear to be interrelated since 50S subunits lacking the 5S RNA complex do not bind tRNA (Dohme & Nierhaus, 1976). Protein S19 can be cross-linked to a tRNA derivative (Ofengand, 1980), and the anticodon of a tRNA can be photochemically cross-linked to the 3′ third of the 16S RNA (Zimmermann et al., 1979).

The evidence summarized in Figure 8 suggests that the interface interactions of RNA and proteins are concentrated in the neighborhood of the 3′-terminal 60% of the 16S RNA and approximately the 3′-terminal half of the 23S RNA. The results presented here greatly extend our picture of the structure of the interface region of the *Escherichia coli* ribosome and emphasize the participation of both protein and RNA interactions in the structure of the subunit interface.

Acknowledgments

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Zinc(II), Cadmium(II), and Mercury(II) Thiolate Transitions in Metallothionein†

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ABSTRACT: The metal-specific absorption envelopes of zinc–, cadmium–, and mercury–metallothioneins and of complexes of these metal ions with 2-mercaptoethanol have been analyzed in terms of Jørgensen's electronegativity theory for charge-transfer excitations by using the spectra of zinc(II), cadmium(II), and mercury(II) tetrahalides as references. By Gaussian analysis the difference absorption spectra of the various forms of metallothionein vs. thionein and of the corresponding 2-mercaptoethanol complexes vs. 2-mercaptoethanol were resolved into three components. For each metal derivative the location of the lowest energy band is in good agreement with the position of the first ligand–metal charge-transfer (LMCT) transition (type $t_2 \rightarrow a_1$) predicted from the optical electronegativity difference of the thiolate ligands and of the central metal ion by assuming tetrahedral coordination. There is also a correspondence between the effects of the metal ion on the position of the first LMCT band and the binding energy of the 2p electrons of the sulfur ligands as found by X-ray photoelectron spectroscopic measurements [Sokolowski, G., Pilz, W., & Weser, U. (1974) *FEBS Lett. 48*, 222]. Due to the lack of exact structural information, the assignment of the two other resolved metal-dependent bands remains conjectural, but it is likely that they include a second LMCT transition (type $t_2 \rightarrow a_1$) analogous to that occurring in tetrahalide complexes of group-2B metal ions. The simplicity of the resolved thiolate spectra and their correspondence to those of tetrahedral models support the view that the various metal-binding sites of metallothionein are chemically similar and that the coordination environment of the metal ion has a symmetry related to that of a tetrahedron [Vašák, M. (1980) *J. Am. Chem. Soc. 102*, 3953].

Metallothionein is a cysteine- and metal-rich protein which was first isolated from the equine renal cortex by Margoshes & Vallee (1957). The same protein has since been found in the liver, kidney, and intestine of numerous other vertebrate species and recently also in a eukaryotic microorganism (Lerch, 1979). All mammalian forms characterized to date contain a single polypeptide chain with 20 cysteinyl residues in a total of 61 amino acid residues and have a chain weight of 6100. The best studied mammalian forms contain a total of 6–7 g-atoms of zinc or of a mixture of zinc and cadmium per mole (Kojima & Kägi, 1978). All metallothioneins are devoid of aromatic amino acids and histidine. The biosynthesis of the protein occurs in the liver, kidney, and intestinal wall and is

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