Cross-Links between Ribosomal Proteins of 30S Subunits in 70S Tight Couples and in 30S Subunits†

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ABSTRACT: Ribosome 70S tight couples and 30S subunits derived from them were modified with 2-iminothiolane under conditions where about two sulfhydryl groups per protein were added to the ribosomal particles. The 70S and 30S particles were not treated with elevated concentrations of NH_4Cl, in contrast to those used in earlier studies. The modified particles were oxidized to promote disulfide bond formation. Proteins were extracted from the cross-linked particles by using conditions to preclude disulfide interchange. Disulfide-linked protein complexes were fractionated on the basis of charge by electrophoresis in polyacrylamide/urea gels at pH 5.5. The proteins from sequential slices of the urea gels were analyzed by two-dimensional diagonal polyacrylamide/sodium dodecyl sulfate gel electrophoresis. Final identification of proteins in cross-linked complexes was made by radioiodination of the proteins, followed by two-dimensional polyacrylamide/urea gel electrophoresis. Attention was focused on cross-links between 30S proteins. We report the identification of 27 cross-linked dimers and 2 trimers of 30S proteins, all but one of which were found in both 70S ribosomes and free 30S subunits in similar yield. Seven of the cross-links, S3–S13, S13–S21, S14–S19, S7–S12, S9–S13, S11–S21, and S6–S18–S21, have not been reported previously when 2-iminothiolane was used. Cross-links S3–S13, S13–S21, S7–S12, S11–S21, and S6–S18–S21 are reported for the first time. The identification of the seven new cross-links is illustrated and discussed in detail. Ten of the dimers reported in the earlier studies of Sommer & Traut (1976) [Sommer, A., & Traut, R. R. (1976) *J. Mol. Biol. 106*, 995–1015], using 30S subunits treated with high salt concentrations, were not found in the experiments reported here.

The three-dimensional structure of the 30S ribosomal subunit has been investigated extensively by a variety of techniques (Brimacombe et al., 1978). Protein cross-linking (Traut et al., 1980; Expert-Bezançon et al., 1977; Sommer & Traut, 1976), immune electron microscopy (Lake, 1980; Stöffler et al., 1980; Kahan et al., 1981), and neutron scattering techniques (Langer et al., 1978; Ramakrishnan et al., 1981) have been used to define ribosomal protein arrangement in the 30S subunit. Preliminary models have been constructed by using this evidence. There is good general agreement of the results from the different experimental approaches. A small number of cross-links have appeared inconsistent with immune electron microscopy (Kahan et al., 1981) and with the neutron scattering model (Ramakrishnan et al., 1981).

Free 30S subunits undergo a magnesium concentration dependent conformational change upon association with 50S subunits (Ball et al., 1973), which requires energy to overcome an activation barrier (Zamir et al., 1971). Chemical modification has been used as a probe of conformational change upon subunit association. Several studies have shown that some proteins are protected from modification by subunit association, while others become more reactive in the 70S ribosome compared to free subunits (Huang & Cantor, 1972; Litman et al., 1976). Similar results have been obtained at specific sites in the 16S RNA of the 30S subunit by using the guanine-specific reagent kethoxal (Herr et al., 1979). Neutron scattering results indicate that the 30S subunit has the same general shape in 70S ribosomes and as free 30S subunits (Stuhrmann et al., 1978; Koch et al., 1978), which suggests that conformation changes that occur when 30S subunits associate with 50S subunits are not drastic structural alterations at this level of resolution.

We have used protein cross-linking with 2-iminothiolane to compare the protein topography of 30S subunits in 70S ribosomal "tight couples" (Hapke & Noll, 1976) and of free 30S subunits derived from the 70S tight couples. Cross-link formation with this reagent would not be expected to be sensitive to small displacements of ribosomal proteins but should detect any major rearrangements or blocking of interface groups by joining of the 30S to the 50S particle. The ribosomes were prepared from slow-cooled mid-logarithmic phase cells to give a ribosome preparation as homogeneous as possible (Noll et al., 1973). The ribosomes were not treated with elevated salt concentrations, a procedure which has been shown to alter protein reactivity (Ghosh & Moore, 1979). Cross-linked proteins were analyzed by two-dimensional polyacrylamide/sodium dodecyl sulfate gel electrophoresis (Kenny et al., 1979), and the final identification of ribosomal proteins in stained spots off the diagonal was made by using the radioiodination technique described by Tolan et al. (1980). The same cross-links were found in the free 30S subunit and in the 70S tight couple. The relative yields appeared similar. We identified 27 cross-linked dimers of 30S proteins and 2 trimers.

The investigation was also undertaken in order to refine and complete the catalog of protein dimers formed between 30S proteins by using 2-iminothiolane since the resolving power of the current methods is better than those used earlier. The evidence for seven of the dimers has not been published previously by this laboratory and is presented here. Ten of the dimers reported in the earlier studies of Sommer & Traut (1976) were not identified in this work. The significance of these findings is discussed as they tighten the correlation of cross-linking with other results on protein topography of the ribosome.

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Experimental Procedures

2-Iminothiolane was from Serva Chemical Co. It was stored over Drierite in a vacuum desiccator at 4 °C. Triethanolamine was purchased from Matheson Coleman and Bell. Acrylamide (practical grade) and *N,N'*-methylenebis(acrylamide) were from Eastman Organic Chemicals and used without further recrystallization. [Bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris)[1] and iodoacetamide were from Sigma, urea (ultrapure) and sucrose (ribonuclease free) were from Schwarz/Mann, 2-mercaptoethanol was from British Drug House, 30% H_2O_2 and LiCl were from Mallinckrodt, KI was from Fischer Scientific, and catalase was from Boehringer Mannheim. Dithioerythritol, Iodo-gen, and Coomassie blue G-250 were purchased from Pierce Chemicals. [^{125}I]Iodine (carrier free, 100 mCi/mL) was purchased from Amersham.

Preparation of Ribosomes and Ribosomal Subunits. Ribosomes were prepared from *Escherichia coli* MRE600 grown in yeast extract/glucose media and harvested in mid-log phase by a slow-cooling procedure to produce "run-off" ribosomes (Noll et al., 1973) as described previously (Lambert & Traut, 1981). The cells were lysed at 0 °C 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 (Hershey et al., 1977). The ribosomes were resuspended in the same buffer except that the $MgCl_2$ concentration was 6 mM, and 70S "tight couples" (Hapke & Noll, 1976) were prepared by zonal centrifugation in the 6 mM $MgCl_2$ buffer with a 10–41% (w/w) sucrose gradient (Lambert & Traut, 1981). The 70S tight couples thus prepared were used without further purification. Ribosomal subunits were prepared from the 70S tight couples by zonal centrifugation using buffers containing 1 mM $MgCl_2$ as described previously (Lambert et al., 1978). In this way, a preparation of 30S subunits was obtained which was free of 50S subunits as judged by sucrose gradient centrifugation.

Cross-Linking of 70S Ribosomes and 30S Subunits and Extraction of Proteins from the Cross-Linked Particles. Ribosomes (3 mg/mL) in 50 mM triethanolamine hydrochloride, pH 8.0, 50 mM KCl, 6 mM $MgCl_2$, and 10 mM dithioerythritol were modified with 12 mM 2-iminothiolane exactly as described previously (Lambert & Traut, 1981). Ribosomal 30S subunits were modified in exactly the same way except that the concentration of $MgCl_2$ was 1 mM. Under these conditions, about two sulfhydryl groups per protein were added to the ribosomal particles (Kenny et al., 1979). The modified particles were incubated with 40 mM H_2O_2 as described previously (Lambert & Traut, 1981; Kenny et al., 1979) to promote disulfide bond formation, many of which form between sulfhydryl groups on different ribosomal proteins. Unreacted H_2O_2 was removed by incubation with catalase (10 μg/mL) for 15 min at 0 °C.

Proteins were extracted from cross-linked 30S subunits by the addition of 1 volume of 200 mM iodoacetamide, 8 M urea, and 6 M LiCl. All free sulfhydryl groups were rapidly alkylated under these conditions, thus preventing the possibility of disulfide interchange (Lambert et al., 1978). After 24 h at 0 °C, the urea/LiCl solution was adjusted to 66% (v/v) acetic acid according to Hardy et al. (1969) to ensure complete precipitation of the RNA. The supernatant proteins were dialyzed against 6% acetic acid and lyophilized. Proteins were extracted from cross-linked 70S ribosomes by using the same procedures. However, it was necessary first to purify cross-linked 70S ribosomes from some ribosome dimers that formed during the cross-linking procedure (Lambert & Traut, 1981; Cover et al., 1981). This was accomplished by centrifugation in linear sucrose gradients [7–25% (w/w)] by using a Beckman SW-27 rotor exactly as described previously (Lambert & Traut, 1981).

Fractionation of Proteins Extracted from Cross-Linked Particles by Polyacrylamide/Urea Gel Electrophoresis. Lyophilized proteins (extracted from about 10 nmol of 70S ribosomes or 30S subunits) were dissolved in 100–150 μL of 8.0 M urea, 40 mM iodoacetamide, and 20 mM Bis-Tris–acetate, pH 3.7, containing pyronine G. The sample was applied to a gel tube (13 cm × 0.6 cm for 70S protein; 10 cm × 0.6 cm for 30S protein) containing 4% (w/v) acrylamide, 0.066% (w/v) bis(acrylamide), 38 mM Bis-Tris, and 6.0 M urea adjusted to pH 5.5 with acetic acid, with an upper gel (0.5 cm × 0.6 cm) of the same composition adjusted to pH 4.7. The reservoir buffers were 20 mM Bis-Tris–acetate at pH 3.7 (upper reservoir) and pH 5.5 (lower reservoir). All gel and buffer solutions contained 1 mM iodoacetamide, a precaution to preclude disulfide interchange (Lambert et al., 1978). Electrophoresis was toward the cathode at 2 mA/tube for 6–8.5 h. The gels were sliced into 0.5-cm fractions, which were stored at −70 °C.

Analysis of Urea Gel Fractions by Two-Dimensional Polyacrylamide/Sodium Dodecyl Sulfate Diagonal Gel Electrophoresis. The proteins in each 0.5-cm slice of urea gel were analyzed exactly as described previously (Lambert & Traut, 1981; Kenny et al., 1979). A detailed description of the diagonal polyacrylamide/sodium dodecyl sulfate gel procedures can be found elsewhere (Sommer & Traut, 1974; Lambert et al., 1978; Kenny et al., 1979; Kenny & Traut, 1979). Slight modifications of those procedures used in this work have been described previously (Lambert & Traut, 1981). The diagonal gels were calibrated by using molecular weights for the ribosomal proteins calculated from their amino acid sequences (Wittmann, 1982): the positions for most of the proteins are shown in Figure 1.

Identification of Components of Cross-Linked Complexes Isolated from Diagonal Gels. The relative mobilities of protein spots resolved in the second dimension of diagonal gel electrophoresis were often insufficient to define uniquely the components, and so the proteins were analyzed further. Stained spots were cut out, and the proteins were eluted and radioiodinated exactly as described previously (Lambert & Traut, 1981; Tolan et al., 1980). The dried radioiodinated proteins, mixed with 100 μg of nonradioactive total 70S protein, were dissolved in 8 M urea, 20 mM Bis-Tris–acetate, pH 3.7, and 1% (v/v) 2-mercaptoethanol and submitted to electrophoresis in two-dimensional polyacrylamide/urea gels as described previously (Lambert & Traut, 1981; Kenny et al., 1979). Proteins were stained with 0.0125% (w/v) Coomassie blue G-250 in 12.5% (w/v) Cl_3CCOOH for 30 min and washed twice with 6% acetic acid before drying onto Whatman 3MM paper for radioautography with Kodak No-Screen medical X-ray film.

Proteins L20 and S12 comigrate in both the diagonal sodium dodecyl sulfate gels and the two-dimensional urea gels. To distinguish between these proteins in the analysis of cross-linked 70S proteins, we used a third polyacrylamide electrophoretic system which separated L20 from S12 as described

[1] Abbreviations: Iodo-gen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Cl_3CCOOH, trichloroacetic acid.

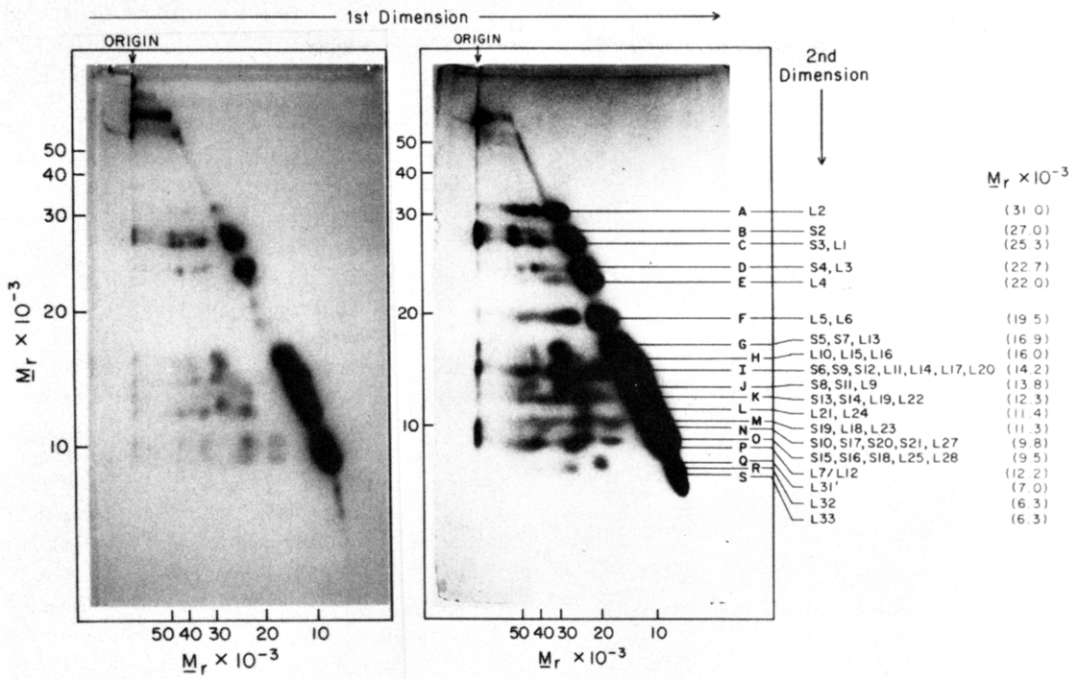

FIGURE 1: Two-dimensional diagonal polyacrylamide/sodium dodecyl sulfate gel electrophoresis of proteins extracted from cross-linked ribosomal particles. (Left panel) Proteins (400 μg) from 30S subunits cross-linked with 2-iminothiolane. (Right panel) Proteins (1 mg) from 70S ribosomes cross-linked with 2-iminothiolane. Electrophoresis in the first dimension [14.5% (w/v) acrylamide] was in the absence of reducing agents, after which the proteins were reduced by incubation of the gel in 3% (v/v) 2-mercaptoethanol and submitted to electrophoresis in a second dimension [17.5% (w/v) acrylamide]. The diagonal techniques have been described in detail and are referred to under Experimental Procedures. Protein cross-links migrate to positions in the first dimension that are related to the sum of the molecular weights of their monomeric components. After reduction of disulfide bonds and electrophoresis in the second dimension, the individual components of a cross-link migrate to positions below the diagonal line of non-cross-linked proteins. The position of each 70S protein on the diagonal was determined as described previously (Kenny & Traut, 1979; Lambert & Traut, 1981). The molecular weight (M_r) calibration for both dimensions was established by using the molecular weights for the ribosomal proteins calculated from their amino acid sequences (Wittmann, 1982), as described by Lambert & Traut (1981). The average molecular weights of the proteins on each of the lettered rows are shown next to the right panel.

previously (Lambert & Traut, 1981; Madjar et al., 1979).

Results

Figure 1 shows diagonal gels of proteins extracted from cross-linked 30S ribosomal subunits (left panel) and cross-linked 70S ribosomes (right panel). Monomeric proteins derived from cross-linked complexes are identified by their coincidence on a single vertical axis below the diagonal line of monomeric non-cross-linked proteins. The sum of the molecular weights of the monomeric components of a cross-linked complex corresponds with the apparent molecular weight of the cross-linked complex determined from its relative mobility in the first dimension of electrophoresis. The positions and molecular weights of the monomeric proteins on the diagonal are shown lettered at the right of Figure 1. It is clear from Figure 1 that the pattern of spots below the diagonal of cross-linked 70S proteins (right panel) is more complicated than that of the diagonal of cross-linked 30S proteins. This was expected since the 70S particle contains 54 proteins while the 30S subunit contains only 21 proteins (Brimacombe et al., 1978).

Fractionation of Cross-Linked Proteins and Analysis of Fractions by Diagonal Gel Electrophoresis. The complexity of the diagonal patterns shown in Figure 1 is evident. The similar mobilities of many monomeric proteins make it difficult to identify unambiguously the protein components of many protein dimers. Many different cross-linked complexes have similar or overlapping molecular weights, and so their monomeric components all fall on the same vertical axis. This makes it difficult to match the protein spots derived from cross-linked complexes. So that these difficulties could be overcome, the mixture of proteins extracted from cross-linked

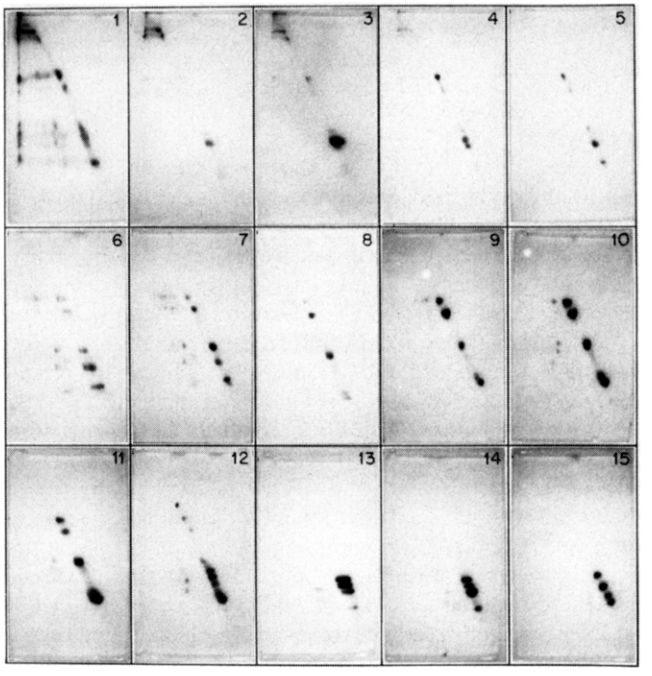

FIGURE 2: Gallery of diagonal gels representing different fractions of proteins extracted from cross-linked 30S subunits. The mixture of proteins (3 mg) was fractionated by polyacrylamide/urea gel electrophoresis at pH 5.5. Each 0.5-cm slice of the urea gel (20 fractions) was analyzed by diagonal gel electrophoresis. The figure shows the first 15 such slices. For further details, see text.

30S subunits, or from cross-linked 70S ribosomes, was fractionated before diagonal gel analysis. The protein mixtures were submitted to electrophoresis in polyacrylamide/urea gels,

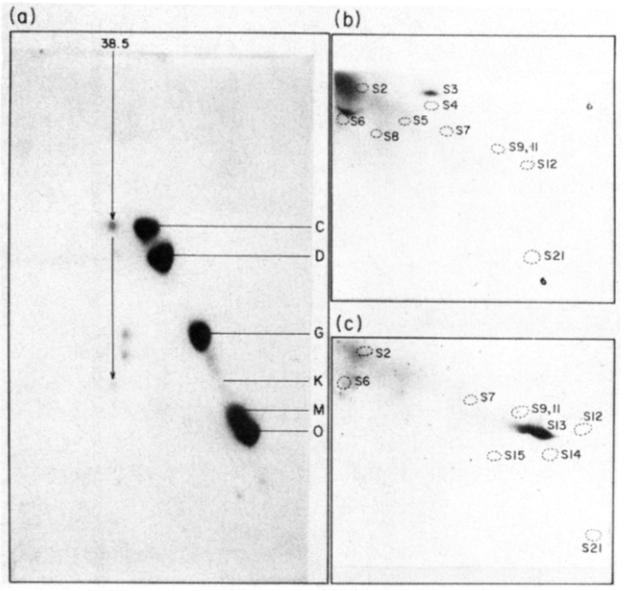

FIGURE 3: Identification of the cross-link S3–S13 in urea gel fraction 10 of proteins extracted from 30S subunits cross-linked with 2-iminothiolane. (a) Diagonal gel pattern of proteins from cross-linked 30S ribosomal subunits, in urea gel fraction 10. Refer to Experimental Procedures for details of protein extraction and fractionation. Proteins that were cross-linked in the first dimension fall below the diagonal line in the second dimension: one such pair of proteins is indicated by the vertical axis numbered 38.5. The number 38.5 is equal to the apparent molecular weight × 10^3 of the cross-linked complex in the first dimension. The horizontal lettered rows correspond with the lettering code of the diagonal of total cross-linked 70S protein shown in Figure 1. (b) Radioautograph of a two-dimensional polyacrylamide/urea gel of radioiodinated protein from the spot 38.5 C. The protein was eluted, radioiodinated, mixed with carrier/marker protein (100 μg of total 70S protein), and submitted to electrophoresis as described in detail under Experimental Procedures. Proteins were stained with Coomassie blue G-250, and the positions of some of the stained 30S proteins are indicated by broken circles drawn on the radioautograph. For a complete ribosomal protein pattern in this gel system, refer to Lambert & Traut (1981). (c) Radioautograph of a two-dimensional polyacrylamide/urea gel of radioiodinated protein from spot 38.5 K.

a procedure which separated the proteins predominantly on the basis of their net charge. The proteins in sequential slices of such a gel were then analyzed by diagonal gel electrophoresis. Figure 2 shows a gallery of diagonal gels representing the first 15 fractions of proteins extracted from cross-linked 30S ribosomal subunits. The fractions not shown (16–20) contained only monomeric proteins of low molecular weight. The fractionation procedure resulted in a substantial simplification of the pattern of spots in each diagonal. The changes in intensity of members of a cross-linked pair in sequential slices also aided in the identification. Similar galleries of diagonal gels representing sequential fractions of proteins extracted from 70S ribosomes cross-linked with 2-iminothiolane or with dithiobis(propionimidate) can be found elsewhere (Lambert & Traut, 1981; Cover et al., 1981).

Several simplified diagonal gels are shown in greater detail in Figures 3–5. Protein spots derived from cross-linked complexes are identified in Figures 3–5 by their coincidence on single vertical axes. Only cross-linked proteins not previously identified (Sommer & Traut, 1976) are so labeled here. Figures 3 and 4 show these cross-links in 30S subunits while Figure 5 shows them in 70S ribosomes. The identity of the other conspicuous protein spots may be found in the earlier publication. Each vertical axis (Figures 3–5) is labeled with the apparent molecular weight × 10^3 of the cross-linked complex in the first dimension, determined for each gel from the relationship between log molecular weight of the proteins and electrophoretic mobility. The spots of each vertical axis indicated by the arrows on the diagonal gels (Figures 3–5) are identified by horizontal lettered rows: the lettering corresponds to that shown in Figure 1. Thus, a typical cross-linked protein dimer is defined by a number (apparent molecular weight × 10^3) and two letters (corresponding to the mobility of the monomeric components in the second dimension).

Identification of a Cross-Linked Protein Pair in Urea Gel Fraction 10 of Proteins Extracted from 30S Subunits. Figure 3a shows the simplified diagonal gel pattern of the proteins in urea gel fraction 10. One of the strong protein pairs is indicated by arrows on the vertical axis labeled 38.5. The horizontal intercepts match a protein spot on row C with a spot on row K. The method for confirming the identity of proteins in the lettered spots is well established, and full details have been published (Tolan et al., 1980). The cross-link 38.5 (row C protein to row K protein) was chosen as an example to illustrate the procedure as used in these experiments.

Row C (Figure 3a) corresponds to the mobility of proteins S3 and L1 (cf. Figure 1). Since the proteins in this diagonal gel were extracted from 30S subunits, then spot 38.5 C must be S3. This was confirmed by elution of the protein, radioiodination, and final identification on two-dimensional polyacrylamide/urea gels. Figure 3b shows a radioautograph of the gel with the positions of some of the 30S marker proteins shown by dotted outlines: S3 was clearly radiolabeled, although on this radioautograph, an artifact was seen near the position of S6 that did not correspond to any ribosomal protein. Row K (Figure 3a) corresponds to the mobility of 30S proteins S13 and S14 (and 50S proteins L19 and L22). Figure 3c shows the spot 38.5 K to be S13. Therefore, we conclude that the cross-link S3–S13 was formed in the intact 30S ribosomal subunit.

Identification of Cross-Links in Urea Gel Fractions 11, 12, and 14 of Proteins Extracted from 30S Subunits. Figure 4a shows one cross-linked dimer on the vertical axis labeled 32.0. The horizontal intercepts match spots on rows G and I. The spot on row G was found to contain only S7 while the spot on row I contained both S9 and S12. The evidence shows that S7 was cross-linked both to S9 (Sommer & Traut, 1976) and to S12. Figure 4b shows one cross-linked pair on the axis labeled 27.0 which matched spots on rows I and K. The spot on row I contained both S12 and S9 while the spot on row K contained S13. We conclude that this pair of spots was derived from two cross-links, S12–S13 (Sommer & Traut, 1976) and S9–S13.

Figure 4c shows two cross-linked pairs identified by the vertical axes labeled 22.0 and 21.5. The spots on rows J and M of pair 22.0 were identified as S11 and S21, respectively. Thus, pair 22.0 was due to the cross-link S11–S21. Spot K of pair 21.5 contained both S13 and S14, while spot M (21.5) contained both S19 and S21. The prominent pair immediately to the left of pair 22.0 (Figure 4c) was from the cross-link S13–S19 (apparent molecular weight of 23.8 × 10^3) previously identified. Thus, 21.5 K–21.5 M cannot represent the cross-link S13–S19, and S13 in spot K of pair 21.5 was likely cross-linked to S21. The presence of S19 in spot M is due to the cross-link S14–S19. An analysis of the molecular weights of the component proteins of the spots of pair 21.5 was consistent with this interpretation.

Identification of Cross-Links in Diagonal Gels of Fractionated Proteins Extracted from 70S Ribosomes. Panels a, b, and c of Figure 5 show diagonal gels of urea gel fractions 12, 13, and 17, respectively, of cross-linked 70S protein

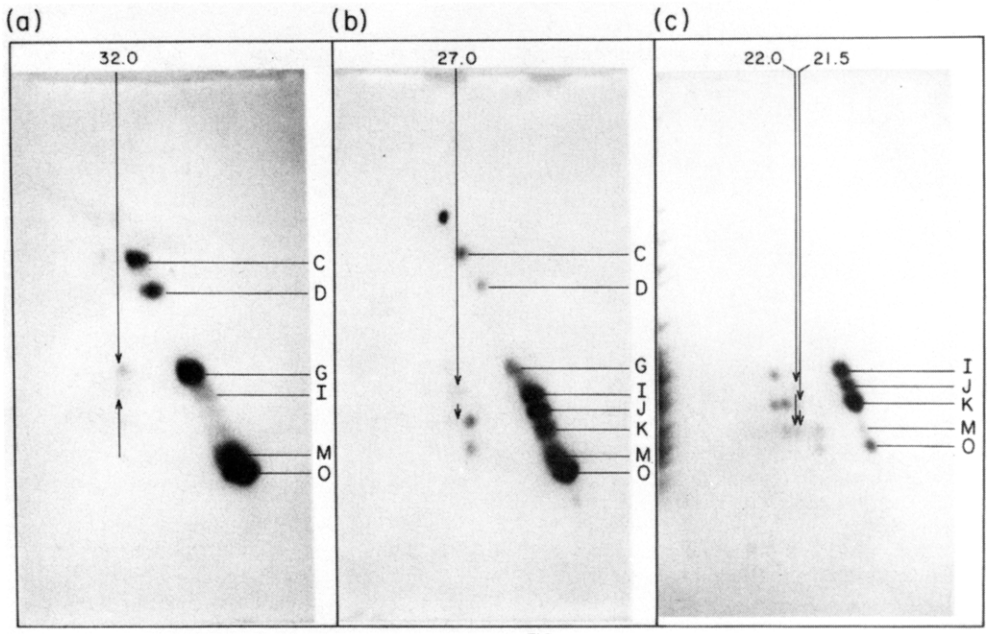

FIGURE 4: Identification of cross-links in urea gel fractions 11, 12, and 14 of proteins extracted from 30S subunits cross-linked with 2-iminothiolane. (a) Diagonal gel pattern of proteins from cross-linked 30S subunits in urea gel fraction 11. (b) Diagonal gel pattern of proteins in urea gel fraction 12. (c) Diagonal gel pattern of proteins in urea gel fraction 14. For further details, see the legend to Figure 3 and the text.

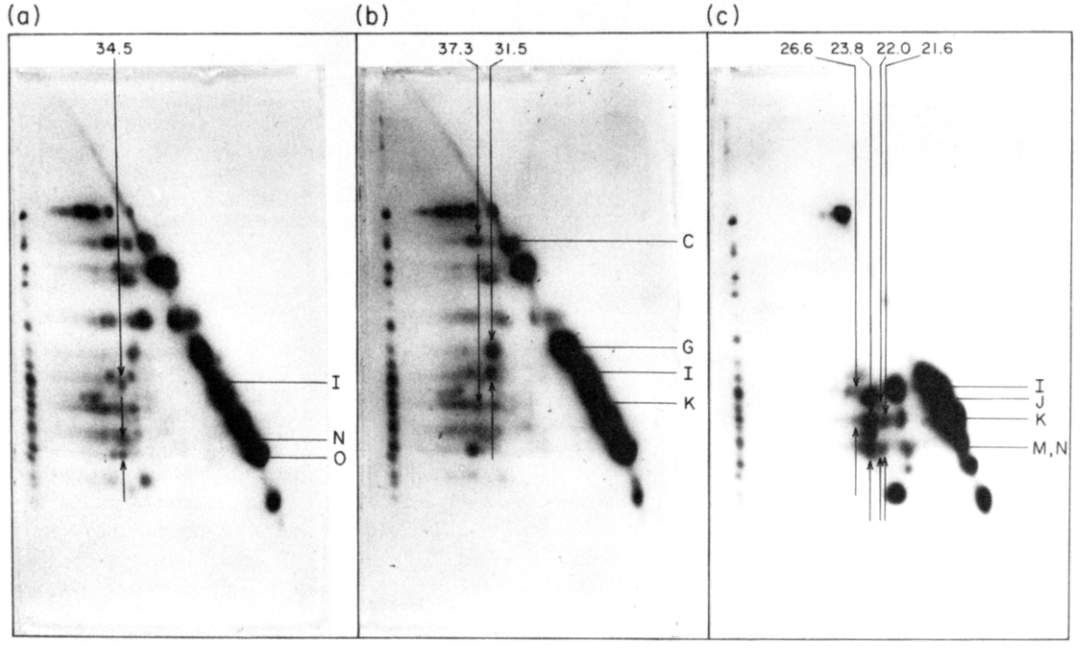

FIGURE 5: Identification of cross-links involving 30S proteins in three urea gel fractions of proteins extracted from 70S ribosomes cross-linked with 2-iminothiolane. Urea gel fractions 12 (a), 13 (b), and 17 (c) were analyzed by diagonal gel electrophoresis. For full details of the urea gel fractionation of proteins from 70S ribosomes, refer to Lambert & Traut (1981). For details of diagonal gel electrophoresis, refer to the legends to Figures 1 and 3.

(Lambert & Traut, 1981). The pairs identified are more conspicuous in this experiment but were also found in 30S subunits. Figure 5a shows a cross-linked complex indicated on the vertical axis 34.5 as the three spots on rows I, N, and O. These spots contained S6, S21, and S18, respectively. The molecular weight additivity criterion indicated that these three proteins formed a disulfide-linked trimer in the intact 70S particle.

Figure 5b shows two vertical axes. The spot on row C of pair 37.3 contained both S3 and L1, while spot 37.3 K contained both S13 and L19. Analysis of the corresponding material from cross-linked 30S subunits showed only S3 and S13. Pair 31.5 matched spots on rows G and I. Spot 31.5 G contained S7 while spot 31.5 I contained both S9 and S12. Thus, pair 31.5 was due to two cross-links, S7–S9 (Sommer & Traut, 1976) and S7–S12.

Figure 5c shows four cross-linked 30S dimers indicated by vertical axes. Spot I of pair 26.6 contained both S12 and S9, while spot 26.6K contained S13. This confirms the presence of the cross-link S12–S13 (Sommer & Traut, 1976) in 70S ribosomes and in addition shows S9–S13. Spot K of pair 21.6 contained both S13 and S14 and was matched with a spot on rows M/N which contained both S19 and S21. Cross-link S13–S19 migrates with an apparent molecular weight of 23.8 $\times 10^3$ and cannot account for the presence of S19 in spot 21.6 M/N. The S19 must derive from the previously identified S14–S19 cross-link. Similar reasoning implicates the presence of a S13–S21 cross-link. So that this conclusion could be

Table I: Identification and Characterization of Cross-Links of 30S Proteins in 30S Subunits and 70S Ribosomes Cross-Linked with 2-Iminothiolane

cross-link	app M_r of cross-link from 30S subunits ($\times 10^{-3}$)[a]	app M_r of cross-link from 70S ribosomes ($\times 10^{-3}$)[a]	sum of M_r's of monomeric proteins ($\times 10^{-3}$)[b]	relative yield[c]
S3-S13	38.5	(37.3)[e]	38.8	H
S7-S12	32.0	31.5[d]	30.7	L
S9-S13	27.0	26.6	27.6	L
S11-S21	22.0	(22.0)[e]	22.1	L
S13-S21	21.5	21.6	21.4	I
S14-S19	21.5	21.6[d]	21.5	I
S6-S18-S21		34.5	33.0	I

[a] The apparent molecular weight (M_r) of each cross-link was determined from its relative mobility in the first dimension of diagonal gel electrophoresis. [b] The molecular weight values used for the monomeric proteins were those calculated from their amino acid sequences (Wittmann, 1982). [c] 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 containing a particular cross-link. The categories H, I, and L are defined as follows: H (high) spots were among the strongest below the diagonal and appeared in three to five successive fractions; L (low) spots were among the weakest spots below the diagonal and only appeared in, at the most, two successive diagonals of urea gel fractions; I (intermediate) spots were below the diagonal of average intensity and usually visible in two or three diagonal gels of urea gel fractions. [d] Cross-links identified in 70S ribosomes cross-linked with dithiobis(propionimidate) (Cover et al., 1981). [e] Cross-links only partially characterized in 70S ribosomes: (L1,S3)-(S13,L19) and S11-(S19,S21).

confirmed, the pair 23.8 (Figure 5c) was found to be the strong cross-link S13–S19 (row K to row M/N). Also, the urea gel fraction following that shown in Figure 5c still contained a cross-linked pair on the vertical axis 21.6: however, the upper and lower spots now contained only S14 and S19, respectively, from the cross-link S14–S19. Pair 22.0 matched a spot on row J, which contained S11, with a spot on row M/N. The latter spot was not resolved from the larger spot 21.6 M/N containing both S19 and S21. Thus, S11 is cross-linked to either S19 or S21, or to both proteins.

Summary of Cross-Links between 30S Proteins Found in 30S Subunits and 70S Ribosomes. Table I lists the six cross-linked dimers and one trimer that had not been identified previously in 30S subunits cross-linked with 2-iminothiolane. Four of the dimers, S7–S12, S9–S13, S13–S21, and S14–S19, are unambiguously shown in both 30S subunits and 70S ribosomes. Evidence for the dimers S3–S13 and S11–S21 is clear in 30S subunits: evidence from the 70S analysis is consistent but insufficient to prove that the dimers form there also. The fact that the molecular weights of the members of dimers add to give a sum close to the apparent molecular weight of the cross-link judged from electrophoretic mobility has been used in past studies to aid in the interpretation of complicated overlapping spots on diagonal gels. The molecular weights for all proteins now available from amino acid sequences (Wittmann, 1982) facilitate this aspect of the analysis of cross-links. The agreement between the two kinds of molecular weight is shown in Table I.

Data such as that shown in Figures 3–5 and Table I were obtained for 27 cross-linked dimers and 2 trimers of 30S proteins in both 30S subunits and 70S ribosomes. Only the data bearing on cross-links not previously reported by this laboratory are illustrated. Examples from both 30S and 70S experiments were shown.

Table II: Summary of Cross-Linked Proteins from the 30S Subunit of the *Escherichia coli* Ribosome

(A) Cross-Links Identified in This Laboratory

highest yield	intermediate yield	low yield
S2-S3[a-d]	S1-S2[e]	S1-S10[e]
S3-S13[a,b]	S1-S18[e]	S2-S8[a,b,d]
S5-S8[a-d]	S2-S5[a,b,d]	S3-S5[a,d]
S6-S18[a-d]	S3-S4[a,b,d]	S4-S8[a,b,d]
S7-S9[a-d]	S3-S9[a,b,d]	S4-S9[a,b,d]
S13-S19[a-d]	S3-S10[a,b,d]	S4-S12[a,b,d]
S18-S21[a-d]	S3-S12[a,b,d]	S7-S12[a-c]
	S4-S5[a-d]	S9-S13[a,b]
	S4-S5-S8[a-c]	S11-S13[a,b,d]
	S4-S13[a,b,d]	S11-S21[a,b]
	S6-S18-S21[b]	
	S7-S13[a,b,d]	
	S12-S13[a,b,d]	
	S13-S21[a,b]	
	S14-S19[a-c]	

(B) Cross-Links Identified in Other Laboratories

S2-S3[i,l,m]	S7-S9[i,k,l]
S2-S5[l]	S7-S13[i]
S2-S8[l]	S7-S13-S19[i]
S3-S10[l]	S8-S15[i]
S4-S5[i,l,m]	S8-S16/S17[i]
S4-S5-S8[i,m]	S9-S13[i]
S5-S8[h-j,l,m]	S11-S18-S21[n]
S5-S8-S15/S16/S17[i]	S13-S19[i,k,l]
S6-S18[i,l]	S14-S19[f]
S6-S14-S18[h]	S18-S21[g,j]

[a] From 30S subunits cross-linked with 2-iminothiolane; this paper. [b] From 70S ribosomes cross-linked with 2-iminothiolane; this paper. [c] From 70S ribosomes cross-linked with dithiobis(propionimidate) (Cover et al., 1981). [d] Sommer & Traut (1974, 1975, 1976). [e] Boileau et al. (1981). [f] Bode et al. (1974). [g] Chang & Flaks (1972). [h] Clegg & Hayes (1974). [i] Expert-Bezançon et al. (1977). [j] Lutter et al. (1972). [k] Lutter et al. (1974). [l] Lutter et al. (1975). [m] Peretz et al. (1976). [n] Shih & Craven (1973).

Discussion

Cross-linked complexes consisting of 30S proteins formed in 30S subunits and in 70S ribosomes were systematically analyzed on diagonal gels of a series of partially purified fractions. The identification of 30S cross-links in 70S ribosomes represents part of an exhaustive study of 70S ribosomes cross-linked by using 2-iminothiolane which includes the characterization of 30S cross-links reported here, 50S cross-links, and 50S–30S interface cross-links reported earlier (Lambert & Traut, 1981). The 30S subunits used in this study were derived in 1 mM Mg from the 70S tight couples used in the study and differ from 30S subunits employed in earlier investigations (Sommer & Traut, 1976) in that the latter were prepared by exposure to high salt concentration.

The 30S cross-links found in 30S and 70S particles are largely the same. Seven cross-links, six dimers and one trimer, were identified that were not identified in the earlier less exhaustive study. These are S3–S13, S7–S12, S9–S13, S11–S21, S13–S21, S14–S19, and S6–S18–S21. In addition to these new cross-links, 21 dimers and 1 trimer found in the earlier study from this laboratory (Sommer & Traut, 1976) were found in both 30S and 70S particles. Table IIA lists all these results and for completeness includes three cross-links to S1 inaccessible to the methods used here (Boileau et al., 1981). Five of the new cross-links, S3–S13, S13–S21, S7–S12, S11–S21, and S6–S18–S21, have not been documented in any earlier reports while S14–S19 and S9–S13 were reported before. The results from other laboratories are listed separately in Table IIB. The present results differ from earlier results

from this laboratory (Sommer & Traut, 1976) in that 10 of the protein pairs reported then with subunits treated with high salt concentration (0.5 M NH_4Cl) were not found in this study in which high salt was avoided. Of the 10 cross-links in question, 4 (S4–S6, S5–S9, S5–S13, and S7–S8) were erroneously assigned due to less effective fractionation procedures then in use. The remaining six dimers (S4–S17, S8–S11, S8–S13, S12–S20, S12–S21, and S13–S17) could not be found in these experiments. The most likely explanation for the discrepancy is the effect of exposure to 0.5 M NH_4Cl on the subunits studied previously. While such treatment is an effective way of removing nonribosomal proteins (Hardy, 1975), it has been shown to alter the reactivity and accessibility of ribosomal cysteine residues toward sulfhydryl reagents (Ghosh & Moore, 1979) and to be associated with a more open conformation of ribosomal particles (C. A. Cowgill, J. W. Kenny, and R. R. Traut, unpublished results). Evidence suggests that enhanced exposure of lysines to modification by 2-iminothiolane could provide additional sites for disulfide cross-linking. The cross-links in question were all in the low yield category.

There is a large and growing measure of agreement among three experimental approaches to the determination of the protein topography of the ribosome: neutron scattering, immune electron microscopy, and cross-linking. Moore has noted three cross-links between proteins whose distance of closest approach from neutron scattering appeared too great to permit cross-linking. These are S3–S12, S4–S9, and S5–S9 (Ramakrishnan et al., 1981). The present study confirms the cross-links S3–S12 and S4–S9 with intermediate and low yields, respectively, in both 30S and 70S particles. The third discrepant cross-link (S5–S9) was erroneously assigned. Lake (1980) has pointed out that the distances between the paired proteins in cross-links S5–S13, S8–S11, and S8–S13 estimated from immune electron microscopy were too great to permit cross-linking. None of the three discrepant cross-links were confirmed in this study. The agreement between cross-linking and the other data, already good, is now improved. This good agreement for the 30S data attests the value of cross-linking data for interpreting the protein topography of the 50S subunit and of the subunit interface for which extensive neutron scattering and electron microscopic data are not yet available.

All but two of the 32 cross-links shown in Table IIA were found in about the same relative yield both in free 30S subunits and in 70S ribosomes. The trimer S6–S18–S21 was only identified in proteins extracted from cross-linked 70S ribosomes. However, the two dimers S6–S18 and S18–S21, that likely make up the cross-linked trimer, were found in good yield in both 70S ribosomes and 30S subunits. The dimer S3–S5, identified previously by Sommer & Traut (1976), was found in low yield in the analysis of proteins extracted from cross-linked subunits but was not found in proteins from cross-linked 70S ribosomes. We consider that small differences in the cross-linking pattern between 30S subunits and 70S ribosomes, among those cross-links of lowest relative yield at the limit of resolution of the analytical technique, are unlikely to be significant. The near identity of the 30S cross-linking pattern of 30S subunits in 1 mM magnesium and 70S ribosomes in 6 mM magnesium is noteworthy. While it is clear that the 30S subunit undergoes a conformational change upon association with 50S subunits (Ball et al., 1973; Zamir et al., 1971; Litman et al., 1976), the results reported here show that the conformational changes are not so large as to change the pattern of protein neighborhoods in the 30S subunit as inferred from protein cross-linking. Protein S1 was also found cross-linked to the same three 30S proteins in both free 30S subunits or in 70S ribosomes (Boileau et al., 1981). It was not found in any "interface cross-links" to 50S proteins in cross-linked 70S ribosomes.

Table II summarizes a large body of information about the protein neighborhoods of the 30S subunit provided by protein cross-linking. Most of the work employed lysine-specific reagents. It appears that we are now near establishing a complete list of cross-links between susceptible 30S protein amino groups: any others will be of low yield. New reagents with different specificities may provide additional information. The next step in the use of cross-linking for the mapping of ribosomal protein topography will be the identification of the cross-linked amino acid residues or cross-linked peptides in protein dimers. The amino acid sequences of all the ribosomal proteins are known (Wittmann, 1982). Certain points of protein cross-linking have already been reported. The cross-linked dimer S18–S21 was formed with *p*-phenylenebis(maleimide) (Chang & Flaks, 1972); the unique cysteine residues in each protein are the most likely point of cross-linking. The amino acid residues cross-linked in the abundant dimer S5–S8 have been identified (Allen et al., 1979). The identification of the specific sites of cross-linking for other neighboring pairs of proteins will greatly extend the resolution of cross-linking as a tool for determining ribosomal protein topography.

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References

Allen, G., Capasso, R., & Gualerzi, C. (1979) *J. Biol. Chem. 254*, 9800–9806.

Ball, L. A., Johnson, P. M., & Walker, I. O. (1973) *Eur. J. Biochem. 37*, 12–20.

Bode, U., Lutter, L. C., & Stöffler, G. (1974) *FEBS Lett. 45*, 232–236.

Boileau, G., Sommer, A., & Traut, R. R. (1981) *J. Biol. Chem. 256*, 8222–8227.

Brimacombe, R., Stöffler, G., & Wittmann, H. G. (1978) *Annu. Rev. Biochem. 47*, 217–249.

Chang, F. N., & Flaks, J. G. (1972) *J. Mol. Biol. 68*, 177–180.

Clegg, C., & Hayes, D. (1974) *Eur. J. Biochem. 42*, 21–28.

Cover, J. A., Lambert, J. M., Norman, C. M., & Traut, R. R. (1981) *Biochemistry 20*, 2843–2852.

Expert-Bezançon, A., Barritault, D., Milet, M., Guerin, M. F., & Hayes, D. H. (1977) *J. Mol. Biol. 112*, 603–629.

Ghosh, N., & Moore, P. B. (1979) *Eur. J. Biochem. 93*, 147–156.

Hapke, B., & Noll, H. (1976) *J. Mol. Biol. 105*, 97–106.

Hardy, S. J. S. (1975) *Mol. Gen. Genet. 140*, 253–274.

Hardy, S. J. S., Kurland, C. G., Voynow, P., & Mara, G. (1969) *Biochemistry 8*, 2897–2905.

Herr, W., Chapman, N. M., & Noller, H. F. (1979) *J. Mol. Biol. 130*, 433–449.

Hershey, J. W. B., Yanov, J., Johnston, K., & Fakunding, J. L. (1977) *Arch. Biochem. Biophys. 182*, 626–638.

Huang, K. H., & Cantor, C. R. (1972) *J. Mol. Biol. 67*, 265–275.

Kahan, L., Winkelmann, D. A., & Lake, J. A. (1981) *J. Mol. Biol. 145*, 193–214.

Kenny, J. W., & Traut, R. R. (1979) *J. Mol. Biol. 127*, 243–263.

Kenny, J. W., Lambert, J. M., & Traut, R. R. (1979) *Methods Enzymol. 59*, 534–550.

Koch, M. H. J., Parfait, R., Haas, J., Crichton, R. R., & Stuhrmann, H. B. (1978) *Biophys. Struct. Mech. 4*, 251–262.
Lake, J. A. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 207–236, University Park Press, Baltimore, MD.
Lambert, J. M., & Traut, R. R. (1981) *J. Mol. Biol. 149*, 451–476.
Lambert, J. M., Jue, R., & Traut, R. R. (1978) *Biochemistry 17*, 5406–5416.
Langer, J. A., Engleman, D. M., & Moore, P. B. (1978) *J. Mol. Biol. 119*, 463–485.
Litman, D. J., Beekman, A., & Cantor, C. R. (1976) *Arch. Biochem. Biophys. 174*, 523–531.
Lutter, L. C., Zeichhardt, H., Kurland, C. G., & Stöffler, G. (1972) *Mol. Gen. Genet. 119*, 357–366.
Lutter, L. C., Bode, U., Kurland, C. G., & Stöffler, G. (1974) *Mol. Gen. Genet. 129*, 167–176.
Lutter, L. C., Kurland, C. G., & Stöffler, G. (1975) *FEBS Lett. 54*, 144–150.
Madjar, J.-J., Arpin, M., Buisson, M., & Reboud, J.-P. (1979) *Mol. Gen. Genet. 171*, 121–134.
Noll, M., Hapke, B., Schreier, M. H., & Noll, H. (1973) *J. Mol. Biol. 75*, 281–294.
Peretz, H., Towbin, H., & Elson, D. (1976) *Eur. J. Biochem. 63*, 83–92.
Ramakrishnan, V. R., Yabuki, S., Sillers, I. Y., Schindler, D. G., Engelman, D. M., & Moore, P. B. (1981) *J. Mol. Biol. 153*, 739–760.
Shih, C. T., & Craven, G. R. (1973) *J. Mol. Biol. 78*, 651–663.
Sommer, A., & Traut, R. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3946–3950.
Sommer, A., & Traut, R. R. (1975) *J. Mol. Biol. 97*, 471–481.
Sommer, A., & Traut, R. R. (1976) *J. Mol. Biol. 106*, 995–1015.
Stöffler, G., Bald, R., Kastner, B., Luhrmann, R., Stöffler-Meilicke, M., Tischendorf, G., & Tesche, B. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 171–205, University Park Press, Baltimore, MD.
Stuhrmann, H. B., Koch, M. H. J., Parfait, R., Haas, J., Ibel, K., & Crichton, R. R. (1978) *J. Mol. Biol. 119*, 203–212.
Tolan, D. R., Lambert, J. M., Boileau, G., Fanning, T. G., Kenny, J. W., Vassos, A., & Traut, R. R. (1980) *Anal. Biochem. 103*, 101–109.
Traut, R. R., Lambert, J. M., Boileau, G., & Kenny, J. W. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 89–110, University Park Press, Baltimore, MD.
Wittmann, H. G. (1982) *Annu. Rev. Biochem. 51*, 155–183.
Zamir, A., Miskin, R., & Elson, D. (1971) *J. Mol. Biol. 60*, 347–364.

Thermodynamic Binding Constants of the Zinc–Human Serum Transferrin Complex†

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ABSTRACT: Serum transferrin is a mammalian iron-transport protein. It has two specific metal-binding sites that bind a variety of metal ions in addition to ferric ion. Equilibrium constants for the binding of zinc(II) have been determined by difference UV titrations using nitrilotriacetic acid and triethylenetetramine as competing ligands. The values are log K_1^* = 7.8 and log K_2^* = 6.4 in 0.10 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid and 15 mM bicarbonate, pH 7.4 at 25 °C. Titrations of the two forms of monoferric transferrin show that K_1^* corresponds to zinc binding to the C-terminal site and K_2^* corresponds to binding at the N-terminal site. These results indicate that at serum bicarbonate concentrations, transferrin should have a higher affinity for zinc(II) than serum albumin and therefore could play some role in zinc transport. A linear free-energy relationship has been constructed which relates the formation constants of a series of zinc(II) and iron(II) complexes. On the basis of the zinc–transferrin binding constants, this relationship has been used to estimate an iron(II)–transferrin binding constant of $10^{7.4}$. Using this ferrous constant and literature values for the ferric transferrin binding constant, one calculates a ferric transferrin reduction potential of −140 mV, which is easily within the range of physiological reductants. Such a result tends to support mechanisms for iron removal from transferrin in which the ferric ion is reduced to the less tightly bound ferrous ion.

Serotransferrin is the primary serum iron-transport protein in mammals. Accordingly, its iron binding properties, as well as those of the closely related proteins ovotransferrin and lactoferrin, have been extensively studied (Chasteen, 1977; Aisen & Listowsky, 1980; Aisen & Liebman, 1972). These proteins all possess two similar, but not identical, specific metal-binding sites, and all have a high binding affinity for iron(III). The transferrins also form less stable complexes with a wide variety of d-block transition metals (Aisen et al., 1969; Casey & Chasteen, 1980; Harris, 1977; Tan & Woodworth, 1969; Gelb & Harris, 1980), main-group metals (Gelb & Harris, 1980; Larson et al., 1978; Harris & Pecoraro, 1983), actinides (Harris et al., 1981; Stevens et al., 1968; Breunger et al., 1969), and lanthanides (Pecoraro et al., 1981; Teuwisson et al., 1972; Meares & Ledbetter, 1977; Luk, 1971). It has been shown that zinc(II) forms fairly stable complexes with both sero- and ovotransferrin (Tan & Woodworth, 1969; Gelb

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