Parameters for the Interaction of Ribosomal Proteins L5, L18, and L25 with 5S RNA from *Escherichia coli*†

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ABSTRACT: Association constants (K_a') for the binding of 50S ribosomal subunit proteins L5, L18, and L25 to the 5S ribosomal RNA of *Escherichia coli* have been determined by a membrane filter assay. Values for K_a' are 2.3×10^8 M^{-1} for the L18–5S RNA complex, 1.5×10^7 M^{-1} for the L25–5S RNA complex, and 2.3×10^6 M^{-1} for the L5–5S RNA complex at 25 °C in TMK buffer (50 mM Tris-HCl (pH 7.6)–20 mM $MgCl_2$–300 mM KCl). Although the affinity of L5 increases by approximately one order of magnitude in the presence of L18, estimation of K_a' was not feasible in the ternary complex. Standard thermodynamic quantities for the individual protein–5S RNA interactions were calculated from the variation of K_a' with temperature. Enthalpy and entropy changes both contribute to the free energy of binding in all three cases. Since the enthalpic terms are small, however, it is unlikely that the associations lead to major alterations in the structure of the ribosomal components. Circular dichroism measurements confirm that the 5S RNA undergoes no detectable change in secondary structure as a result of association with L5 or L25. Formation of the L18–5S RNA complex, by contrast, is accompanied by a significant increase in the circular dichroism at 268 nm, suggesting that the protein induces a shift in the configuration of one of the double-stranded regions of the RNA molecule. This observation may help to explain the strong cooperative influence of L18 upon the binding of L5 to the 5S RNA.

There is now considerable evidence that 5S RNA plays a pivotal role in the structure and function of 50S ribosomal subunits from prokaryotic cells. When 5S RNA is removed from mature 50S particles, or omitted from reconstituted subunits, their activity in protein synthesis sharply declines and they exhibit deficiencies in their normal complement of structural proteins (Erdmann et al., 1971; Dohme & Nierhaus, 1976). Functions in which the 5S RNA has been implicated include the binding of aminoacyl-tRNA to the ribosomal A site as well as several ribosome-linked enzymatic activities (Erdmann et al., 1973; Dohme & Nierhaus, 1976; reviewed by Erdmann, 1976). Stable incorporation of 5S RNA into the ribosomal particles of *Escherichia coli* is dependent upon the presence of 50S subunit proteins L5, L18, and L25 and it has been suggested that all four components become integrated as a unit (Yu & Wittmann, 1973).

The binding of L5, L18, and L25 to the 5S RNA has been investigated by a variety of methods, ranging from sucrose gradient centrifugation and polyacrylamide gel electrophoresis to membrane filter retention and chromatography on agarose–5S RNA affinity columns (Horne & Erdmann, 1972; Gray et al., 1973; Yu & Wittmann, 1973; Burrell & Horowitz, 1975). Each of the proteins associates independently with the RNA molecule (Yu & Wittmann, 1973; Gray et al., 1973; Feunteun et al., 1975) and it has recently been demonstrated that the stoichiometry of all of the complexes at saturation is near 1:1 under optimal conditions (Spierer & Zimmermann, 1978). The complete primary structures of the three proteins and of the 5S RNA are known (Brosius et al., 1975; Dovgas et al., 1975; Chen & Ehrke, 1976; Brownlee et al., 1967) and nucleotide sequences to which the proteins bind have been described (Gray et al., 1973; Zimmermann & Erdmann, 1978). Interactions involving L5 and L18 have also been found to possess a strongly cooperative character (Feunteun et al., 1975; Spierer & Zimmermann, 1978) that is strikingly reflected in the ability of the ternary L5, L18–5S RNA complex to associate with 23S RNA (Gray et al., 1972; Spierer et al., 1978). Since the 5S ribonucleoproteins are well defined, convenient to manipulate, and endowed with a number of interesting properties, they comprise a particularly fertile experimental system for studies on the mechanism of specific protein–nucleic acid interactions.

Here we report the use of a sensitive membrane filter assay to determine the apparent association constants for the formation of complexes between the 5S RNA and each of the three proteins, L5, L18, and L25, from *E. coli*. Estimation of the variation of these constants with temperature enabled us to calculate standard thermodynamic parameters for the interactions. We have also assessed the consequences of protein binding on 5S RNA configuration from measurements of circular dichroism. Our findings indicate that there are wide differences in the affinities of the proteins for 5S RNA but that structural changes resulting from the associations are relatively limited. It has been possible nonetheless to correlate the binding of L18 with alterations in a base-paired portion of the 5S RNA molecule.

Materials and Methods

Ribosomal Components. Conditions for the growth of *E. coli* MRE600, for the labeling of cellular RNA and proteins with radioactive precursors, and for the isolation of ribosomal particles have been described (Muto et al., 1974). Ribosomes to be used for the preparation of 5S RNA were first freed of contaminating tRNA by sedimentation through a cushion of 30% sucrose in 10 mM Tris-HCl (pH 7.6)–1 mM $MgCl_2$–100

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mM KCl (Spierer & Zimmermann, 1978). The washed particles were then extracted with phenol and the 5S RNA was separated from the large ribosomal RNAs by sucrose gradient centrifugation. Each preparation of 5S RNA was checked for conformational homogeneity by polyacrylamide gel electrophoresis in the presence of 1 mM $MgCl_2$ (Richards et al., 1973); only those containing over 95% A form were used in binding experiments. Unlabeled and [^{3}H]amino acid labeled proteins were isolated from purified 50S ribosomal subunits with 67% acetic acid and fractionated by ion-exchange chromatography on phosphocellulose and carboxymethylcellulose (Zimmermann & Stöffler, 1976). The specific radioactivity of the ^{3}H-labeled proteins was approximately 2500 counts/min per μg; the specific acitivity of ^{14}C- and ^{32}P-labeled 5S RNA ranged from 2×10^3 to 2×10^6 counts/min per μg. Sequence molecular weights for proteins L5, L18, and L25 are 20 172 (Chen & Ehrke, 1976), 12 770 (Brosius et al., 1975), and 10 912 (Dovgas et al., 1975), respectively; that for 5S RNA was calculated to be 38 853 from its base composition (Brownlee et al., 1967).

Analysis of Protein-RNA Complexes by Filter Assay. From 0.004 to 40 μg (0.1 to 1000 pmol) of ^{32}P- or ^{14}C-labeled 5S RNA was mixed with an equimolar quantity of unlabeled or labeled L5, L18, or L25 in 100 μL of TMK buffer (50 mM Tris-HCl (pH 7.6)–20 mM $MgCl_2$–300 mM KCl), and incubated for 30 min at the temperature indicated in the text. Reaction mixtures were then filtered through nitrocellulose membranes (Millipore, type HA, 13 mm) previously soaked in TMK buffer for 2 h under vacuum. Filtration was carried out at the incubation temperature with a flow rate of 0.5 mL/min. The filters were washed with 100 μL of TMK buffer, dried in air, and analyzed for radioactivity. In the absence of 5S RNA, about 65% of the protein applied to the filter was retained throughout the concentration range examined. The retention of protein was reduced by small amounts of urea, however, and reproducible results were obtained only when the concentration of this substance was kept below 0.2 M. All filtration data for protein–5S RNA complexes were corrected for nonspecifically adsorbed RNA, which was routinely less than 5% of the RNA added, by the analysis of parallel mixtures lacking protein. The specific retention of 5S RNA in the presence of protein was taken as 100% when the fraction of RNA immobilized on the filter attained its maximum plateau value. This value represented a recovery of about 35% of the RNA added to the incubation and defined the retention efficiency (Riggs et al., 1970). A small decline in retention efficiency at component concentrations above 10^{-6} M was noted in experiments with L5 and corrections were made accordingly.

Measurement of Circular Dichroism. Samples containing 39 μg (1 nmol) 5S RNA, 61 μg (3 nmol) of L5, 19 μg (1.5 nmol) of L18, and 22 μg (2 nmol) of L25, either alone or in various combinations, were prepared in 1 mL of TMK buffer and incubated for 15 min at 30 °C. CD[1] spectra of individual proteins, of 5S RNA, and of protein–5S RNA complexes were measured between 215 and 305 nm with a Cary Model 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment. The temperature of the cell holder was maintained by a thermostatically controlled circulating water system. The CD spectra of the protein–RNA complexes were corrected for the presence of excess protein by subtracting the appropriate portion of the curve for the free protein(s) from the experimental curves for the mixtures. Mean residue ellipticities, [θ], were calculated per mole of amino acid or nucleotide monomer. Since the circular dichroism of L5, L18, and L25 was undetectable from 240 to 305 nm, a mean nucleotide molecular weight of 322 was used for the 5S RNA, whether free or bound, throughout this region. For the calculation of CD spectra from 215 to 240 nm, mean residue weights for L5, L18, L25, L5–5S RNA, L18–5S RNA, L25–5S RNA, and L5, L18, L25–5S RNA were taken to be 113, 109, 114, 197, 227, 231, and 162, respectively, on the basis of amino acid and nucleotide composition (Chen & Ehrke, 1976; Brosius et al., 1975; Dovgas et al., 1975; Brownlee et al., 1967).

[1] Abbreviation used: CD, circular dichroism.

Results

Determination of Association Constants. In the course of studies on the stoichiometry of complexes containing 5S RNA and 50S ribosomal subunit proteins L5, L18, and L25 by sucrose gradient centrifugation, it became apparent that the three proteins differ widely in their affinity for the RNA molecule (Spierer & Zimmermann, 1978). Moreover, L18 was found to cooperatively stimulate the binding of L5 at component concentrations roughly an order of magnitude less than were required for the interaction of L5 alone. These findings prompted us to undertake a quantitative analysis of protein–5S RNA complex formation and to attempt the correlation of protein binding with structural changes in the 5S RNA molecule.

The sucrose gradient technique is unsuitable for the measurement of association constants because the analysis is carried out under nonequilibrium conditions and results in a substantial dilution of the reactants. A membrane filter assay was therefore exploited to estimate the amount of 5S RNA associated with a given quantity of protein in an equilibrium mixture of the two components (Riggs et al., 1970; Yarus & Berg, 1970; Yu & Wittmann, 1973). The assay requires that L5, L18, and L25 each bind quantitatively to the membranes, but that 5S RNA not be retained unless it is specifically combined with one of the proteins. In addition, filtration itself must perturb the equilibrium as little as possible. The accuracy of the measurements is not directly dependent upon retention efficiency, however, as long as a fixed proportion of the complexes remains bound to the filters throughout the concentration range tested (Riggs et al., 1970). Control experiments indicated that these conditions were reasonably well met by our procedures.

Apparent association constants (K_a') for each of the complexes were determined as follows. The fraction of radioactively labeled 5S RNA retained on a filter was first measured as the concentration of both RNA and protein was varied over a wide interval. These values were next expressed as a percentage of the 5S RNA specifically retained at saturation and plotted vs. component concentration as in Figure 1. Since there is a single binding site for each protein in the 5S RNA (Spierer & Zimmermann, 1978) and since the incubations contain equimolar amounts of protein and RNA, then the concentrations of free RNA, of free protein, and of the complex should be the same when the retention of 5S RNA on the filter is one-half its maximum value. The apparent association constants in this case are simply $([R]/2)^{-1}$ or $([P]/2)^{-1}$, where R represents total RNA and P is total protein. Values of K_a' in TMK buffer at 0 °C were calculated from the data of Figure 1 according to these premises and are presented in Table I. They demonstrate that the affinity of L18 for the 5S RNA is about 16 times greater than that of L25 and nearly 100 times as great as that of L5. Measurement of the association constant for L5 in the presence of L18 was not possible because the binding of 5S

TABLE I: Thermodynamic Parameters for the Protein-5S RNA Interactions.

complex	K_a'[a] ($M^{-1} \times 10^{-6}$) at 0 °C	K_a'[b] ($M^{-1} \times 10^{-6}$) at 25 °C	$\Delta G^{\circ\prime}$[c] (kcal/mol) at 25 °C	$\Delta H^{\circ\prime}$[d] (kcal/mol)	$\Delta S^{\circ\prime}$[e] (eu)
L18-5S RNA	309	225	−11.4	−2.7	29.2
L25-5S RNA	18.7	14.9	−10.4	−1.5	29.8
L5-5S RNA	3.75	225	−8.8	−2.0	22.8

[a] Assuming that each protein binds to a single site in the 5S RNA, equilibria should conform to the equation: $K_a' = [PR]/([P-PR][R-PR])$, where P is total protein, R is total 5S RNA, and PR is the complex. Since protein and RNA are present in equimolar amounts, $[PR] = [P-PR] = [R-PR] = [P]/2 = [R]/2$ when half of each component has entered the complex. K_a' should therefore be equal to $([P]/2)^{-1} = ([R]/2)^{-1}$ when RNA retention on the filter is half-maximal. Values in table were calculated accordingly from data of Figure 1. [b] Values for K_a' at 25 °C were obtained from data of Figure 2 by interpolation. [c] Standard Gibbs free energy changes at 25 °C were calculated as: $\Delta G^{\circ\prime} = RT \ln K_a'$. [d] Standard enthalpy changes were calculated from slopes of the plots in Figure 2 according to the van't Hoff equation: $[\partial \ln K/\partial(1/T)]_P = -\Delta H^{\circ\prime}/R$. [e] Standard entropy changes were obtained from the relation: $\Delta G^{\circ\prime} = \Delta H^{\circ\prime} - T\Delta S^{\circ\prime}$.

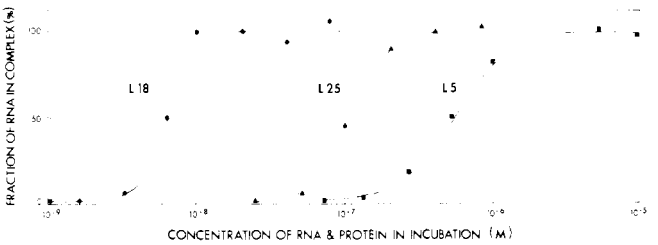


FIGURE 1: Retention of protein-5S RNA complexes on nitrocellulose membrane filters. Equimolar amounts of 5S RNA and ribosomal proteins L5, L18, or L25 were incubated at various concentrations in TMK buffer and filtered, as described in Materials and Methods. Incubation, filtration, and washing were all performed at 0 °C. Results are expressed as a percentage of the maximum fraction of RNA bound to the filter once the plateau was reached. The fraction of added RNA retained at saturation (retention efficiency) was generally about 35%. Data were corrected for RNA bound in the absence of protein, which was always less than 5% of the input RNA. (■—■) L5-5S RNA; (●—●) L18-5S RNA; (▲—▲) L25-5S RNA.

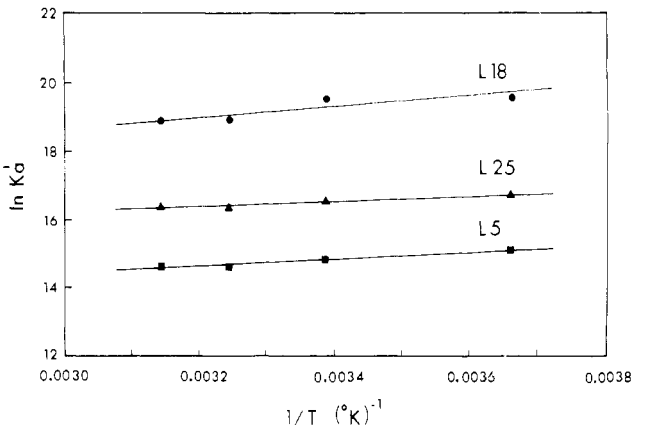


FIGURE 2: Dependence of association constants on temperature. Apparent association constants at 22, 35, and 45 °C were obtained from curves analogous to those of Figure 1. The straight lines were fitted according to the method of least squares and have the form $\ln K_a' = M(T)^{-1} + C$. (■—■) L5-5S RNA; (●—●) L18-5S RNA; (▲—▲) L25-5S RNA.

RNA to the filter was governed chiefly by the latter protein.

Determination of Thermodynamic Parameters. Association constants for the binding of L5, L18, and L25 to the 5S RNA were determined at 22, 35, and 45 °C by the procedures described in the preceding section. The experimental values of $\ln K_a'$ are plotted as a function of $(1/T)$ in Figure 2, along with those previously obtained at 0 °C. The values of K_a' at 25 °C were derived by interpolation and used to calculate the standard free energy changes of complex formation (Table I). Since the variation of $\ln K_a'$ with $(1/T)$ is linear between 0 and 45 °C, the standard enthalpy changes may also be calculated from the data in Figure 2 by use of the van't Hoff equation. These figures, as well as the corresponding entropy changes, are listed in Table I. Values for all three interactions are similar and, in particular, the small negative enthalpies in each case would argue against the disruption of an appreciable number of noncovalent bonds in these processes. It is of interest that the thermodynamic parameters for the protein-5S RNA complexes are comparable to those for the interaction of $tRNA^{Ile}$ with its cognate synthetase (Yarus & Berg, 1970) suggesting that their mechanisms may be very much alike.

Circular Dichroism of 5S RNA and Protein-5S RNA Complexes. We considered the possibility that certain features of protein-5S RNA association, such as the cooperative stimulation of L5 binding by L18, could be ascribed to alterations in the secondary structure of the interacting molecules. The configuration of the 5S RNA both in the free state and in complexes with L5, L18, and L25 was therefore investigated by the measurement of circular dichroism between 215 and 305 nm. The portion of the spectrum above 240 nm is of particular importance. While the contribution of proteins to the circular dichroism in this region can be disregarded, nucleic acids possess a large positive band near their absorption maximum whose position and amplitude are strongly dependent upon their secondary structure (Yang & Samejima, 1969).

The CD spectrum of 5S RNA and of its complex with L18 is illustrated in Figure 3. Although the wavelength maximum of the main positive band is 268 nm in both cases, the amplitude is 20–25% higher in the L18-5S RNA complex than in free 5S RNA. This difference must be due exclusively to a change in the secondary structure of the RNA since the circular dichroism of the protein at 268 nm is negligible. The association of L18 also produces a slight increase in the magnitude of the small negative band at 298 nm. Interestingly, the CD spectrum of the L18-5S RNA complex between 215 and 240 nm is not simply the sum of the spectra of L18 and 5S RNA alone (Figure 3). Although this anomaly may also result from an alteration in 5S RNA structure, it is difficult to interpret because both components are optically active in the relevant portion of the spectrum. It is noteworthy that the profiles for both free 5S RNA and for the L18-5S RNA complex are invariant from 3 to 30 °C.

The CD spectra of the L5- and L25-5S RNA complexes do not deviate by more than 2–3% from the spectrum of free 5S RNA above 240 nm and, within experimental error, they can be accounted for by summing the appropriate protein and

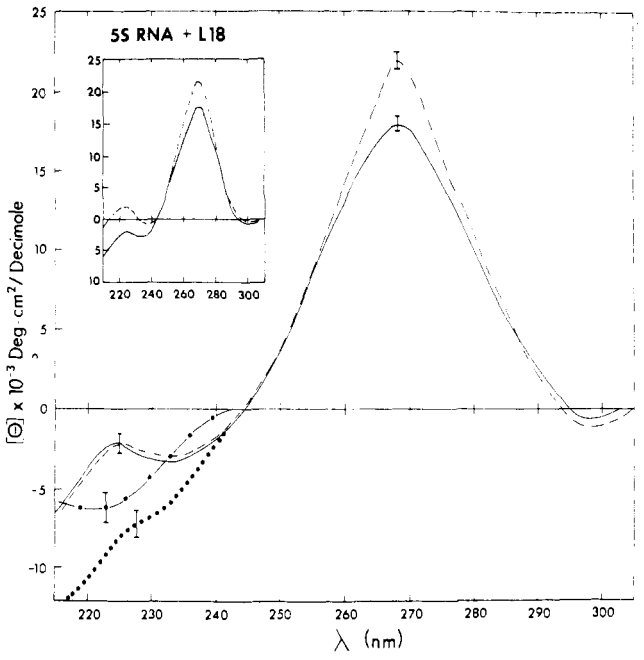


FIGURE 3: CD spectra of free 5S RNA, free L18, and L18-5S RNA complex. Spectra of 5S RNA (—), protein L18 (●—●), and L18-5S RNA complex (- - -) were measured at 23 °C in TMK buffer as described in Materials and Methods. Spectrum calculated by summing data for L18 and 5S RNA alone (●●●). Error bars indicate the average deviation of ellipticities in two duplicate experiments with different complexes. Reproducibility of wavelengths of extrema was ±1 nm. Insert shows the spectrum of free 5S RNA (—) and of 5S RNA in the L18-5S RNA complex (- - -): the latter curve was obtained by subtracting the spectrum of L18 from the experimentally determined spectrum of the complex.

5S RNA curves in the 215–240-nm region (data not shown). These observations suggest that the attachment of L5 and L25 does not lead to any significant shifts in the secondary structure of either protein or RNA. Finally, the presence of all three proteins in the complex produces no greater change in the CD spectrum of the 5S RNA than L18 alone (data not shown), indicating that the entire difference is attributable to binding of the latter protein.

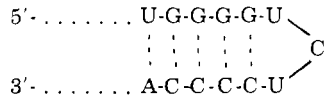
Discussion

We have measured the association constants, standard thermodynamic parameters, and circular dichroism spectra for complexes of the 5S RNA with each of the 50S ribosomal subunit proteins L5, L18, and L25. Values of K_a' span almost two orders of magnitude, with L18 exhibiting the highest affinity and L5 the lowest. All three complexes were previously shown to contain about one molecule of protein per molecule of RNA at saturation (Spierer & Zimmermann, 1978), and we found no evidence to support the earlier suggestion that the molar ratio of L18 to 5S RNA reaches 2:1 (Feunteun et al., 1975). Our estimate of approximately 10^8 M^{-1} for the K_a' of the L18-5S RNA interaction indicates a much greater stability than do previous values of 10^7 and 10^6 M^{-1} obtained by fluorescence and X-ray scattering techniques, respectively (Feunteun et al., 1975; Österberg & Garrett, 1977). Reasons for these discrepancies are unknown, although the various procedures employed are quite different in principle. The range of values of K_a' for the protein-5S RNA complexes reported here is very similar to that determined for the binding of 30S subunit proteins S4, S8, and S20 to the 16S ribosomal RNA (Spicer et al., 1977). It is likely that all of these associations are strengthened substantially by cooperative interactions involving other subunit constituents during ribosome assembly.

The standard free energy changes for protein-5S RNA complex formation are all large and negative, reflecting the strength of the three interactions. Although both enthalpy and entropy appear to contribute to binding, the estimated values of ΔH must be interpreted with caution since the variation of $\ln K_a'$ is slight and could merely reflect a temperature-dependent artifact in the assay procedure. These measurements nonetheless support the view that no major structural rearrangements occur in any of the reactants as a result of their mutual association and are consistent with the CD data, at least insofar as concerns the 5S RNA. We therefore infer that there is a reasonably good fit between complementary sites in the 5S RNA, on the one hand, and in proteins L5, L18, and L25, on the other, when the components are in their native configurations.

The binding of L18 produces an appreciable increase in the circular dichroism of the 5S RNA, both in the major positive band at 268 nm and in the minor negative band at 298 nm. No differences were observed in the spectra of complexes containing either L5 or L25, however. Our findings for L5 and L18 are in agreement with similar measurements by Bear et al. (1977) but we have not confirmed the small decrease of the main CD band in the presence of L25 that they report. The CD changes resulting from the interaction of L5, L18, and L25 together, which have also been studied by Fox & Wong (1978), do not differ significantly from those provoked by L18 alone.

Interpretation of the circular dichroism of RNA molecules is not without pitfalls but, although the significance of the negative band near 300 nm remains unclear, certain properties of the main positive band have been correlated with specific structural features (Yang & Samejima, 1969). In particular, the magnitude of the main band has been attributed primarily to the strength of base-stacking interactions, whereas its position is believed to reflect the proportion of base pairs, varying from about 260 nm for a perfect double helix to about 275 nm for a completely single-stranded molecule. However, even in RNAs with a constant number of base pairs, a red shift can be expected to follow a selective increase of stacking interactions within single-stranded segments owing to the enhanced ellipticity of the unpaired bases at 275 nm. Since the association of L18 with the 5S RNA alters the amplitude of the 268-nm band, but not the position of its maximum, the difference is unlikely to result from increases in either base pairing or base stacking. Our interpretation thus differs from that of Bear et al. (1977) who, on the basis of comparable CD alterations, postulated that L18 brings about the increase of secondary, or even tertiary, structure in the RNA molecule. It is more probable that the change derives from the perturbation of a structural regularity within existing double-stranded portions of the 5S RNA, such as the tilt of the bases, the orientation of the ribose moieties, or the disposition of the ribose-phosphate backbone itself (Yang & Samejima, 1969). One candidate for the affected region lies between residues 82 and 94:



This loop has been implicated in the binding of L18 by partial ribonuclease digestion of the L18-5S RNA complex (Gray et al., 1973; Zimmermann & Erdmann, 1978) and is conserved in a wide variety of prokaryotic 5S RNAs (Fox & Woese, 1975).

We are not yet able to account for the specificity of association between L18 and the 5S RNA, although it must ultimately reside in the sequence and conformation of the interacting molecules. Low-angle X-ray scattering studies suggest that both protein and RNA are highly elongated (Österberg et al., 1976a,b). The positively charged N terminus of L18 may therefore be accessible for binding to the 5S RNA (Österberg & Garrett, 1977) much as has been suggested for the association of histone with DNA (Weintraub & van Lente, 1974) where rather similar changes in CD have been observed (Adler et al., 1975). Although recent evidence suggests that the N-terminal residues of L18 may not be essential for L18–5S RNA association (Newberry et al., 1978), electrostatic interactions between basic amino acid side chains and negatively charged phosphate groups would undoubtedly contribute additional stability to the complex and are compatible with the substantial binding entropies involved. Another intriguing question is raised by the cooperativity of interaction among L5, L18, and 5S RNA. While the stimulation of L5 binding by L18 may arise from direct association between the two proteins, it could also be mediated by changes in the RNA. Specifically, the structural perturbations reflected in the CD spectrum of the L18–5S RNA complex might increase the accessibility of bases in helical portions of the nucleic acid molecule to ligands such as L5. Although these considerations are admittedly speculative, it seems likely that the binding sites for L5 and L18 are closely related to one another both structurally and functionally (Spierer & Zimmermann, 1978).

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