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Physical Characterization of a Ribosomal Nucleoprotein Complex†

Thomas R. Tritton*,‡ and Donald M. Crothers

ABSTRACT: The complex between ribosomal protein L24 and its RNA binding site (that region of the 23S RNA which the protein protects from ribonuclease digestion) has been studied by various physicochemical methods. The RNA is composed of two fragments of about 160 and 140 nucleotides which interact with each other to form the L24 binding site. Circular dichroism spectroscopy suggests that the two interacting fragments have a unique region of secondary structure which is not present in either of the two components alone; hence there are important structural interactions between regions of the RNA which are separated in the primary sequence. Addition of the L24 protein to the RNA site promotes a structural change associated with base unstacking, but with little or no change in the hydrogen-bonded base pairing. Heat activation is not required for complex formation. Thermal denaturation studies reveal a broad featureless transition and the amount of hypochromic change indicates that the RNA site contains less secondary structure than other RNAs such as tRNA and total rRNA. Temperature-jump relaxation measurements on the mechanism of unfolding of the RNA show a concerted melting of the entire secondary and tertiary structure, which is altered upon addition of the protein. A structural basis for this RNA-protein complex is discussed.

The *Escherichia coli* ribosome is made up of 55 different protein and 3 different RNA species. Highly specific interactions between these macromolecules are required for assembly and subsequently for function of the ribosome in protein synthesis. In general three types of interactions are possible: protein-protein, RNA-RNA, and protein-RNA. The topography of protein-protein interactions has been probed by a number of techniques, the most revealing of which have been the use of chemical cross-linking agents, affinity analogues, and specific antibodies. A recent review (Traut et al., 1974) demonstrates that a self-consistent picture of protein-protein interactions is rapidly emerging. Knowledge of the specific interactions among the RNA species and the arrangement of the RNA in the ribosomal architecture is very limited, although the nucleotide sequences of the three RNAs are either known (5S RNA; Brownlee et al., 1968) or in an advanced stage of analysis (16S and 23S RNA; Fellner, 1974). Our understanding of the nature of the RNA-protein interactions is at a more intermediate stage of development. About 20 of the 55 proteins bind specifically and individually (i.e., in the absence of one or more other ribosomal proteins) to one of the rRNAs. The stoichiometry of interaction and the approximate location of the specific binding region in the intact RNA sequence have been established for these proteins (reviewed by Zimmermann, 1974). In addition the solution conditions

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06520. *Received March 29, 1976.*

‡ Present address: Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510.

necessary for stable interaction between proteins S4 and L24 with, respectively, 16S RNA and 25S RNA have been determined (Schulte et al., 1974).

A biophysical approach to the understanding of specific rRNA-protein interactions is made difficult by the extremely large size of the intact 16S and 23S RNA. Thus it is advantageous to simplify the system by isolating specific binding sites for individual proteins within the RNA structure. There is actually no functional meaning for the term site; it is generally used to refer to a region of the RNA resistant to RNase digestion in the presence of the protein, although these sites are certainly functionally significant units within the total ribosome structure. Known RNA sites range in size from about 40 nucleotides for protein S8 (Schaup et al., 1973) to 300 or more nucleotides for S4 (Schaup et al., 1971) and L24 (Branlant et al., 1973). Isolation of such sites allows one to investigate both the nature of the site itself and its interaction with its specific protein.

In this paper we will discuss spectroscopic and kinetic studies of a ribonucleoprotein complex consisting of *E. coli* ribosomal protein L24 and its corresponding RNA site. This complex is inherently interesting in its own right as a model of RNA-protein interactions in the ribosome. In addition, protein L24 (and presumably its RNA site) is not simply a structural protein in the 50S subunit but is also a part of the functional unit which comprises the binding site for the growing polypeptide being synthesized on the ribosome (Eilat et al., 1974). The complex is relatively easy to isolate (Crichton and Wittmann, 1973) by sequential trypsin and RNase digestion of intact ribosomes, the RNA and protein each acting to protect the other from hydrolytic attack. Originally the RNA moiety was thought to consist of a single species of about 100 nucleotides; further investigations, however, revealed that L24 actually protected from nuclease digestion multiple RNA fragments of a few hundred nucleotides (Branlant et al., 1973). The major bulk of the RNA, however, consists of two contiguous (or nearly so) fragments of about 160 and 140 nucleotides and we have concentrated on the interaction of these two RNAs with L24.

The nature of the problem is to elucidate the secondary and tertiary structural characteristics of the RNA site and the RNA-L24 complex. In particular, we are interested in the conformation of the isolated RNA and how it changes when bound to protein. An especially intriguing problem is the question of how a small protein like L24 (molecular weight ~15 000) affords protection against nuclease digestion to a much larger bulk of RNA (total molecular weight ~100 000). A similar situation exists in the S4-16S RNA interaction and probably in other protein-RNA complexes as well and may thus be of some general biological significance.

Materials and Methods

Growth of Cells and Isolation of Ribosomes. E. coli MRE600 was grown in a 100-l. New Brunswick fermentor in L broth (1% tryptone, 0.5% yeast extract, 0.8% NaCl, 0.1% glucose, adjusted to pH 7.4 with NaOH). The cells were grown to late log phase, chilled on ice, and harvested in a Sharples continuous-flow centrifuge. Following two wash cycles with buffer A, the cells were frozen in small packets for later use. Ribosomes were prepared according to Staehelin et al. (1969) and were stored in small aliquots at 1000 A_{260} units/ml in liquid nitrogen.

Buffers and Chemicals. All chemicals were reagent grade; sucrose used in ribosome preparations was Mann "ultrapure". Buffer A[1] is 0.1 M NH_4Cl-10 mM magnesium acetate-20 mM Tris-HCl (pH 7.5)-0.5 mM EDTA-3 mM 2-mercaptoethanol. The isolated RNAs and L24-RNA complexes were stored in a buffer containing 30 mM Tris-HCl (pH 7.4), 0.35 M KCl, 10 mM $MgCl_2$, and 6 mM 2-mercaptoethanol (binding buffer). This buffer was chosen to optimize the binding of L24 to the RNA as determined from the data of Schulte et al. (1974). Mercaptoethanol was dialyzed out before thermal denaturation or temperature-jump experiments as high temperature promotes a decomposition process leading to a sharply increasing ultraviolet absorbance.

TMA buffer is 5 mM Tris-HCl (pH 8.0)-10 mM magnesium acetate. The gel extraction buffer (PCEP) is 1 mM sodium phosphate (pH 7.0)-10 mM sodium cacodylate-1 mM EDTA-160 mM sodium perchlorate.

Sephadex G-75 was from Pharmacia; CM-cellulose and TEAE-cellulose were Sigma products. Ribonuclease A and trypsin were Worthington enzymes.

RNA Gel Electrophoresis. Analytical disc gels (5 × 75 mm run at 2 mA per tube) and preparative slab gels (20 × 40 cm run at 25 mA) were 10% acrylamide and 0.2% bisacrylamide (Eastman). Purified tRNA and 5S RNA were used to estimate molecular weights. The gels were stained with methylene blue. The RNA bands were cut from the slab gels and homogenized in PCEP buffer in a tissue grinder tube. The homogenate was vigorously shaken for 1 h and then spun in a Sorvall RC2-B refrigerated centrifuge at 2000*g* for 10 min. The pellet was reextracted in the same manner and the two supernatants were combined and twice passed through a 0.45 μm Millipore filter to remove methylene blue. The RNA so obtained can then be dialyzed into an appropriate buffer. Equivalent results were also obtained by direct electrophoretic extraction of the stained gels.

Protein Gel Electrophoresis. Two-dimensional gel electrophoresis of ribosomal proteins was performed according to Kaltschmidt and Wittmann (1970).

Melting Curves. Thermal transitions of RNA and RNA-protein complexes were measured on a Cary 14 spectrophotometer as described previously (Cole et al., 1972). The melting temperature (T_m) was taken as the temperature at which the absorbance had risen halfway between its initial (A_i) and final (A_f) valves. The extent of transition is

$$\beta = 1 - \frac{A_f - A_T}{A_f - A_i}$$

where A_T is the absorbance at a particular temperature and the percent hypochromicity is

$$H = \frac{A_f - A_i}{A_f}(100)$$

Circular Dichroism. CD spectra were recorded at ambient temperature in a Cary 60 spectropolarimeter equipped with a 6001 CD attachment. Results are calculated in terms of molar ellipticity

$$\phi = 100\psi/lm$$

[1] Abbreviations used are: rRNA, ribosomal RNA; tRNA, transfer RNA; RNase, ribonuclease; A_{260} unit, that amount of RNA dissolved in 1 ml which gives an absorbance of 1.0 in a 1-cm path length; Na_2EDTA, disodium ethylenediaminetetraacetic acid; TEAE-cellulose, triethylaminoethylcellulose; CD, circular dichroism; Tris, tris(hydroxymethyl) aminomethane; CM, carboxymethyl; buffer A, 0.1 M NH_4Cl-10 mM magnesium acetate-20 mM Tris-HCl (pH 7.5)-0.5 mM EDTA-3 mM 2-mercaptoethanol; TMA, 5 mM Tris-HCl (pH 8.0)-10 mM magnesium acetate; PCEP, 1 mM sodium phosphate (pH 7.0)-10 mM sodium cacodylate-1 mM EDTA-160 mM sodium perchlorate.

where ψ is the measured ellipticity in degrees, l is the pathlength in centimeters, and m is the RNA phosphate concentration in moles per liter.

Temperature-Jump Measurements. The T-jump instrument is a split-beam modification of the original Eigen-DeMayer configuration (Crothers, 1971). Experiments were carried out and analyzed as described by Crothers et al. (1974).

RNA and Protein Determinations. RNA concentration was estimated by assuming that a 0.1% solution had an absorbance at 260 nm of 20. The molecular weights of the two RNA fragments are estimated from gel mobilities to be 56 000 (160 nucleotides) and 49 000 (140 nucleotides). Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Isolation of L24-RNA Complex. We have substantially modified the procedure originally reported for purifying the L24-RNA complex (Crichton and Wittmann, 1973) and thus report it in detail here. 2000 A_{260} units of 70S ribosomes in TMA buffer were digested with 1 mg of trypsin for 20 h at 37 °C. Two-dimensional gel electrophoresis confirmed the finding of Crichton and Wittmann (1973) that all the ribosomal proteins except L24 are hydrolyzed by this procedure. The resulting L24-rRNA–peptide mixture was chromatographed on a Sephadex G-75 column (2.5 × 50 cm) by eluting with TMA at 20 ml/h. The RNA-L24 peak is eluted in the void volume and can be used to purify further either the L24 protein or the RNA binding site.

L24 Protein. We have found that it is quite easy to obtain pure L24 from its RNA complex (obtained as above) by batch chromatography on TEAE-cellulose in 7 M urea–5 mM Tris-HCl (pH 7.0). Urea is routinely used in the purification of ribosomal proteins and does not cause irreversible structural changes. The urea dissociates the complex and the low ionic strength promotes tight binding of the RNA, but not L24, to the TEAE-cellulose. At least a tenfold excess (TEAE to phosphate equivalents) of the resin is stirred for 12 h with the L24-RNA complex and the L24 supernatant is recovered by centrifugation. Greater than 99% of the RNA (as judged by ultraviolet absorbance) is removed to yield essentially pure L24 protein.

L24 RNA Binding Site. The RNA binding site is defined as that RNA which is not digested by RNase in the presence of the protein. The usual procedure is to digest the complex with RNase in solution and remove the proteins by phenol extraction. We have found this procedure gives variable results and usually leaves residual (but active) RNase in the sample. To overcome these problems we have employed CM-cellulose-bound RNase (prepared according to Cahn et al., 1970) for nuclease digestion. This nuclease activity can be removed by a simple centrifugation step. We have also found that this procedure gives reproducibly well-defined gel patterns. The insolubilized RNase has a specific activity of about 200 units/g. The nuclease digestion was carried out for 45 min at 0 °C using about 1 g of insoluble enzyme per 1000 A_{260} units of RNA. The RNase is removed by centrifugation and L24 by phenol extraction and the RNA digest is lyophilized (in our experience with small RNAs, lyophilization does not cause irreversible structural changes as long as buffer and salt are present; however, we cannot rule out this possibility in the present case). This is then dissolved in a small amount of TMA and applied in 50-A_{260}-unit aliquots to slab gels. The 160- and 140-nucleotide fragments are the major high-molecular-weight products and are eluted from the gel and dialyzed into the binding buffer. These RNA species are derived from the extreme 5′ end of the 23S RNA and are probably contiguous in the primary sequence (Branlant et al., 1973). Our nomenclature is RNA-3A for the 140-nucleotide fragment and RNA-1 for the 160 nucleotide fragment, consistent with the fingerprinting work of Branlant et al. (1973). One-to-one stoichiometric mixtures of the two RNA fragments are called recombined RNA. We cannot be sure that our fragments are exactly the same as those of Branlant et al. (1973) but in each case the isolation procedure selects that RNA which is protected from RNase by L24. Consequently we assume that the bulk features of the RNAs are the same in both instances.

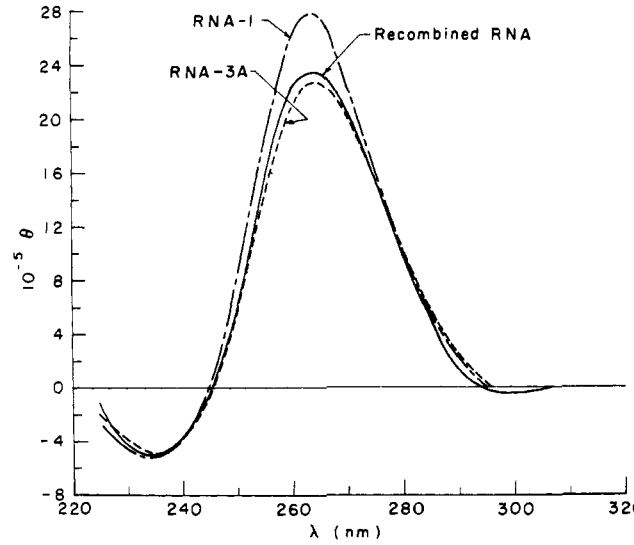

FIGURE 1: Circular dichroism spectra of the RNA species in binding buffer.

Results

Circular Dichroism Studies. Figure 1 shows the CD spectra of RNA-1, RNA-3A, and the recombined RNA at 1:1 stoichiometry. In all three cases there is a maximum in the molar ellipticity at about 265 nm and a minimum of about 235 nm as expected for polymeric RNA molecules. The wavelength maximum is the same in each case but the total ellipticity varies between the three RNA samples, indicating some variations in the amount of secondary structure. Notably, the recombined RNA shows a negative trough at 295 nm which is not detected in either of the component RNAs (see also Figure 2C).

In Figure 2 we see the effect on the RNA CD spectra of adding a threefold molar excess of purified L24. A control spectrum showed that the protein contributes nothing to the RNA spectrum at this concentration. Both RNA-1 and RNA-3A show slight shifts to longer wavelengths; this effect is not apparent with the recombined RNA. In all three cases the negative band at 235 nm shows a decrease in ellipticity in the presence of protein. The large positive band at 265 nm is unchanged in amplitude for either RNA-1 or RNA-3A alone when protein is added, but the recombined RNA shows a slight decrease in this region in the presence of L24. Again, the weak negative band at 295 nm is only detected in the recombined RNA; the ellipticity of this band decreases, but not to zero, when L24 is present.

The reconstitution of a functional 30S subunit from its component RNA and protein requires a heat-activation step (Traub and Nomura, 1969). This is probably necessary to overcome an activation barrier of one of the components to achieve the correct conformation for assembly. Also, Schutle et al. (1974) showed a similar heat requirement for the binding

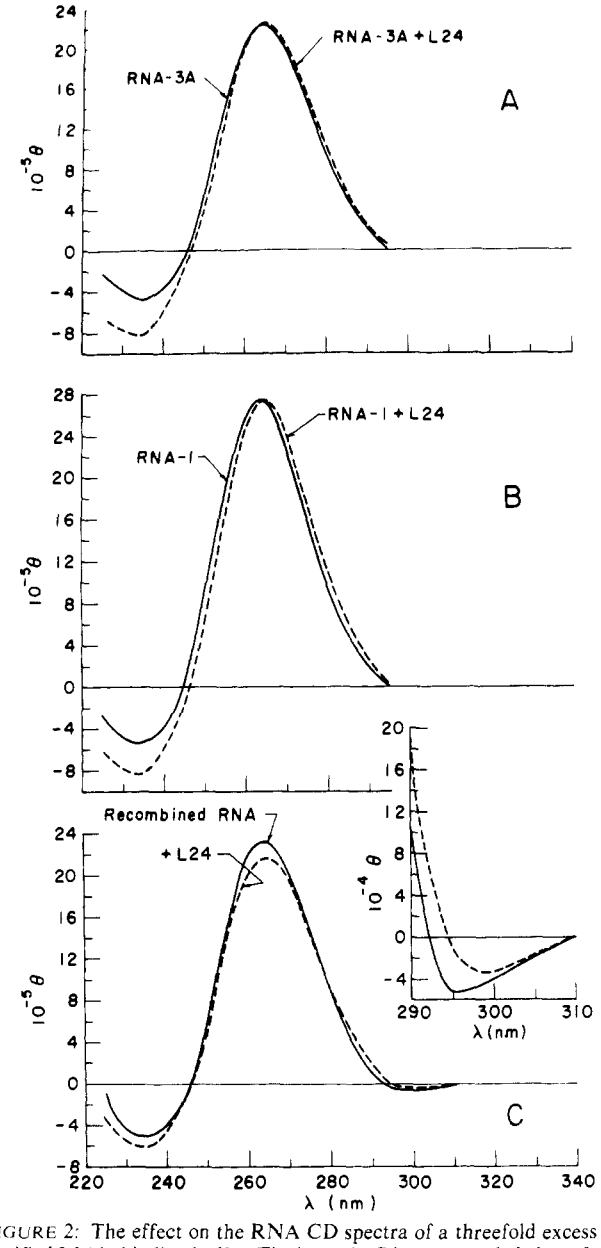

FIGURE 2: The effect on the RNA CD spectra of a threefold excess of purified L24 in binding buffer. The insert in C is an expanded view of the negative 295-nm band which is only present in recombined RNA.

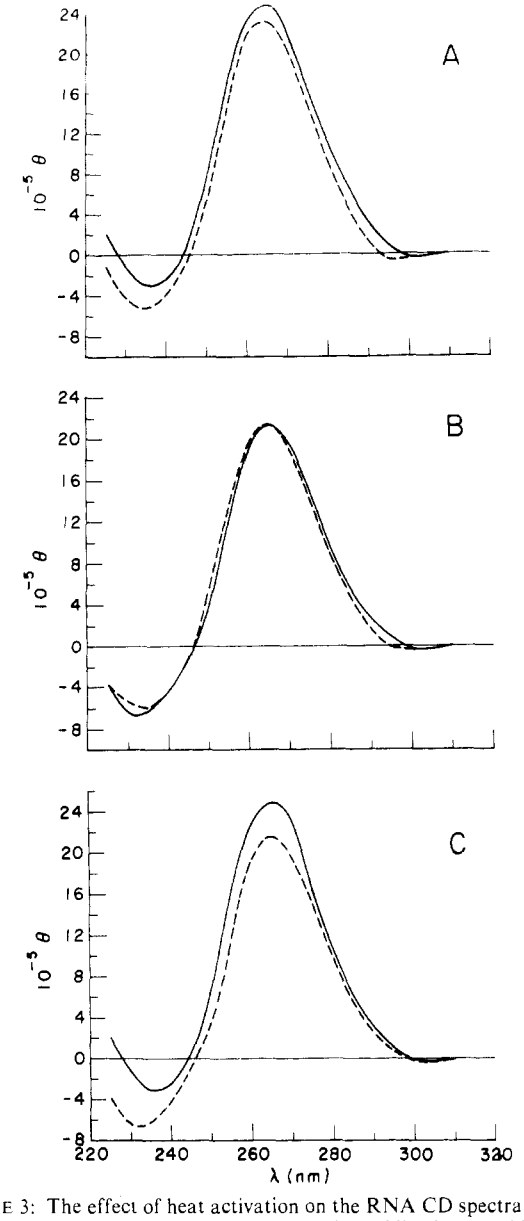

FIGURE 3: The effect of heat activation on the RNA CD spectra of recombined RNA in binding buffer. (A) The dashed line is control RNA; the solid line is heat-activated RNA at 37 °C for 1 h. (B) The dashed line is control RNA + L24, and the solid line is heat-activated RNA + L24., (C) The dashed line is heat-activated RNA + L24, and the solid line is heat-activated RNA alone.

of L24 and S8 to *intact* 23S and 16S RNA, respectively. We have investigated the effect on the CD spectra of the L24-RNA site of heat activation. We show results for the recombined RNA, but qualitatively similar results were obtained for both RNA-1 and RNA-3A. Figure 3A reproduces the spectra of recombined RNA before and after a heat activation at 37 °C for 1 h. Both the bands at 265 and at 235 nm have increased ellipticity upon heat activation. In addition the negative trough at 295 shows a decreased ellipticity when the RNA is heated. Figure 3B, on the other hand, shows that, when L24 is added prior to heat activation, the CD spectrum is generally unchanged upon heating. This suggests that addition of the protein may provoke a similar structural change in the RNA to that produced by heat activation. The heat activated recombined RNA itself, however, does show a different spectrum in the presence of L24 as shown in Figure 3C.

Melting Studies. Thermal denaturation profiles of the two RNA species, recombined RNA, and their combinations with a threefold excess of L24 are shown in Figure 4. In all cases the transitions are broad and featureless, with the melting process occurring over essentially the whole temperature range of 20–100 °C. This contrasts the observation by Schulte et al. (1974) that intact 23S RNA does not show the onset of melting until about 48 °C. The total fragment mass is less than 10% of the 23S RNA mass, however, and melting of the 5′ end of the molecule might not produce a detectable optical change in the whole 23S RNA. The T_m values calculated for each case are shown in Table I along with the percent hypochromicity values. The characteristic transition temperatures are about the same for each RNA and are slightly increased upon addition of L24. The hypochromicity values are somewhat lower than either intact rRNA or tRNA (generally around 20%). RNA-1 shows essentially no change in hypochromism upon addition of L24, whereas RNA-3A and the recombined RNA show respectively a small increase and decrease when the protein is present. In the absence of Mg^{2+} ions, the melting curve shows even less hypochromism and a lower T_m (Table

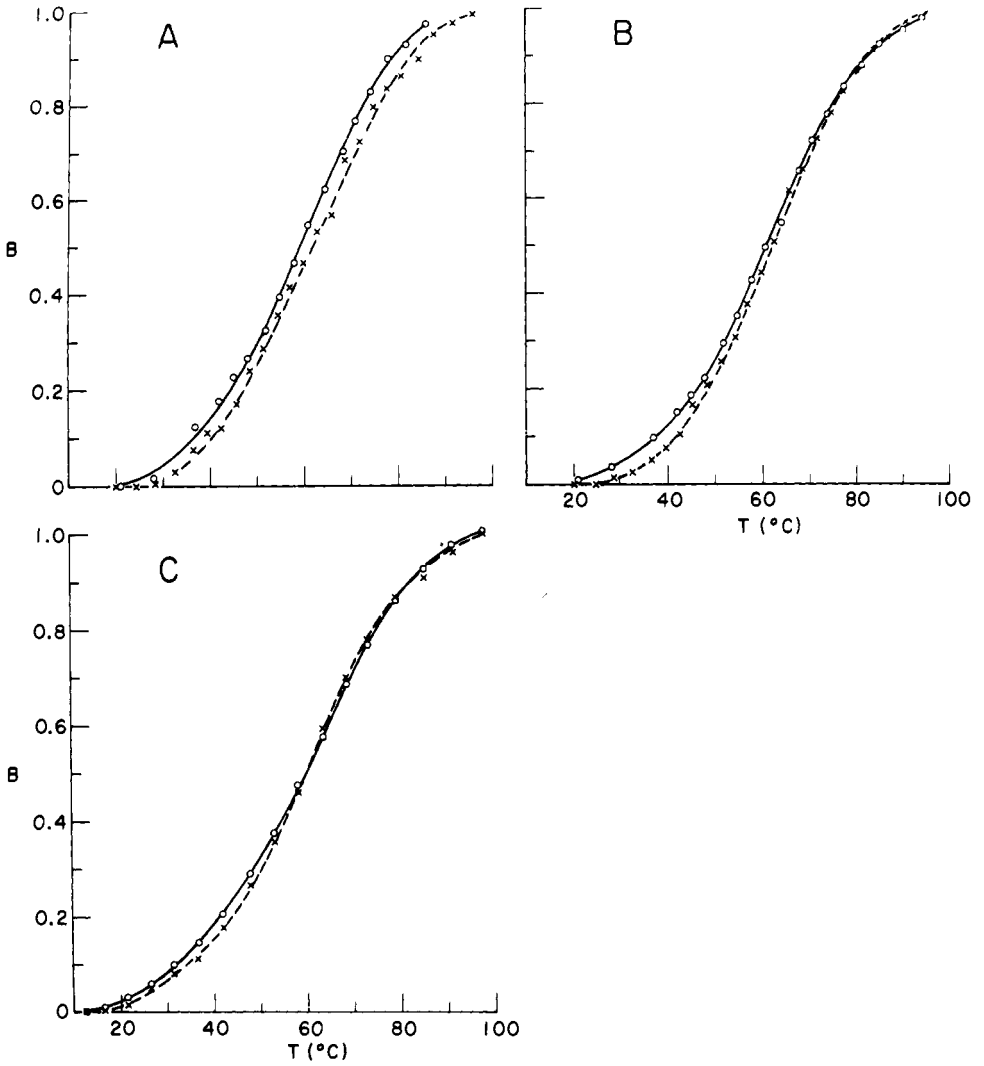

FIGURE 4: Thermal denaturation profiles on the indicated RNA (O) and RNA + L24 (X) in binding buffer. (A) RNA-3A; (B) RNA-1; (C) recombined RNA.

TABLE I: Summary of Melting Studies.[a]

	RNA Alone		RNA + L24	
	T_m	% H	T_m	% H
1. RNA-1	61.5	17.2	62.5	17.1
2. RNA-1, no Mg^{2+}	56.0	14.9	52.5	15.1
3. RNA-3A	59.5	16.0	61.5	16.8
4. Recombined RNA	59.0	17.6	59.5	15.8

[a] In each instance, the data were obtained as described under Materials and Methods in 30 mM Tris-HCl (pH 7.4)–0.35 M KCl–10 mM $MgCl_2$. In case 2, the Mg^{2+} was removed by exhaustive dialysis prior to melting; the actual curves in this case are not shown.

I), indicating that Mg^{2+} ions are required for the stabilization of structure as expected.

Temperature-Jump Studies. Because of the featureless nature of the melting profiles only a minimum of structural information is available from them. A more definitive insight into the structural events of the thermal denaturation process is provided by the temperature-jump method (Crothers et al., 1974). With this technique we measure the time dependence of the various components of the melting process and construct a melting curve which is the derivative of the usual melting curve. From this information we can draw conclusions on the nature of the thermal unfolding process and thus on the structure which led to it.

Figure 5 shows the differential melting curves for the two separate RNA fragments, recombined RNA, and each in the presence of L24. These curves are constructed by plotting the amplitude of the observed temperature-jump relaxations as a function of temperature with the maximum in each curve being defined as the melting temperature (T_m). Each RNA (except RNA-3A) and RNA–protein case shows two resolvable relaxations and thus two maxima indicating that the melting process occurs in at least two discrete steps. Figure 6 shows the relaxation times corresponding to the points in the differential melting curves. Both of these relaxation times are orders of magnitude slower than generally observed for the melting of simple hairpin helices (Crothers et al., 1974; Gralla and Crothers, 1973a), and thus we assign them to a concerted melting of the entire secondary and tertiary structure of the RNA (see Discussion).

In the simple case of all relaxation processes being well separated on both the time and temperature scales, it is convenient to think of each relaxation as corresponding to an all-or-none transition between two states

$$A \overset{\tau}{\rightleftarrows} B \qquad (1)$$

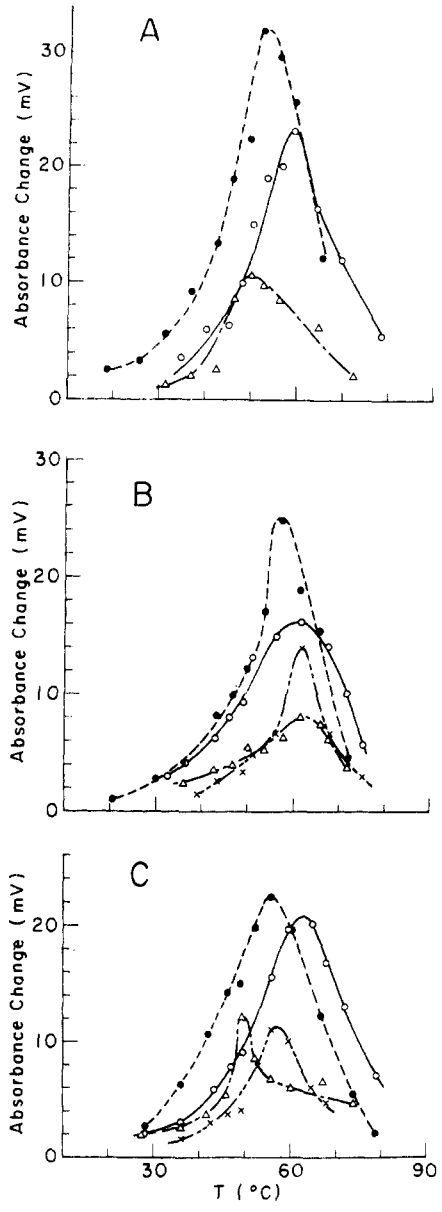

FIGURE 5: Differential melting curves for the various RNAs and L24-RNA complexes in binding buffer. The absorbance change is plotted vs. the temperature corresponding to the midpoint of the temperature-jump size (generally 4.6 °C). The amplitudes were measured at 266 nm. (A) RNA-3A: (●) RNA + L24, slow τ; (○) RNA alone, slow τ; (△) RNA + L24, fast τ. (B) RNA-1: (●) RNA + L24, slow τ; (○) RNA alone, slow τ; (△) RNA + L24, fast τ; (X) RNA alone, fast τ. (C) Recombined RNA: (●) RNA + L24, slow τ; (○) RNA alone, slow τ; (△) RNA + L24, fast τ; (X) RNA alone, fast τ.

A straightforward analysis (Gralla and Crothers, 1973b) then allows one to calculate the kinetic and thermodynamic quantities for the system. In the present instance the two relaxations are separated by about an order of magnitude on the time axis but only by a few degrees on the temperature scale. Thus the relaxations are coupled and cannot be treated independently and the melting process being observed must be described by the presence of at least three states

$$A \overset{\tau_1}{\rightleftarrows} B \overset{\tau_2}{\rightleftarrows} C \qquad (2)$$

Taking the approximation $\tau_2 \ll \tau_1$ and using standard methods of analysis (Crothers, 1971) the kinetics are described by two equations

$$\frac{1}{\tau_1} = k_1 + k_{-1}\left[\frac{K_2}{1+K_2}\right]$$

$$\frac{1}{\tau_2} = k_2 + k_{-2}$$

where the constants are defined by

$$[A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} B \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} C]$$

$$K_1 = \frac{k_1}{k_{-1}}$$

$$K_2 = \frac{k_2}{k_{-2}}$$

It is then possible to construct Arrhenius plots for each individual rate constant because we know that at the T_m (assumed to be the maxima in Figure 5)

$$k_i = k_{-i}$$

and that at high temperature

$$\tau \approx 1/k_i$$

The enthalpy changes are then calculated from the difference in the corresponding activation energies for k_i and k_{-i}. Other schemes are possible but we have analyzed the data by this means because it is the simplest coupled mechanism and allows us to compare the results of RNA melting in the presence and absence of L24. A summary of all the relevant kinetic and thermodynamic data is contained in Table II.

Discussion

Circular Dichroism Studies. The CD spectra allow us to conclude that the RNA fragments in the L24 binding site interact with each other. RNA-1, RNA-3A, and recombined RNA show the characteristic positive (265 nm) and negative (235 nm) bands but only in the recombined RNA is the negative band at 295 nm apparent. This weak intensity band occurs in tRNA, whole ribosomes, and ribosomal RNA, very weakly in DNA, but not in single-stranded homopolymers (Tinoco and Cantor, 1970). The 295-nm band is overlapped strongly by the 265-nm band, and its magnitude and position are very sensitive to slight shifts in the larger transition, but the effect is believed to be associated with the formation of base-paired conformations. If this is true, then the recombined RNA has a unique region of secondary structure which is not present in either of the components. In any event, the two fragments must interact with each other. This is similar to the recent observation (Ungewichell et al., 1975) that the RNA comprising the binding site for ribosomal protein S4 undergoes structural interactions between two regions of the RNA that are separated in the primary sequence. We cannot be sure (since there is no functional assay) that isolated rRNA sites maintain the same conformation as in the intact ribosome. We do know, however, that the appropriate ribosomal protein (L24 in this case) binds specifically to its isolated site, just as it does in the intact ribosome (Zimmermann, 1974), suggesting that the RNA conformation is similar to that in the whole ribosomal particle. Thus the 23S RNA (at least the 5′ end) is probably not simply an array of hairpin helices made from contiguous regions in the primary chain, but must be folded on itself in such a way as to allow interactions between separated regions.

Addition of the L24 protein to the RNAs causes some structural changes to occur, but the CD spectral changes are

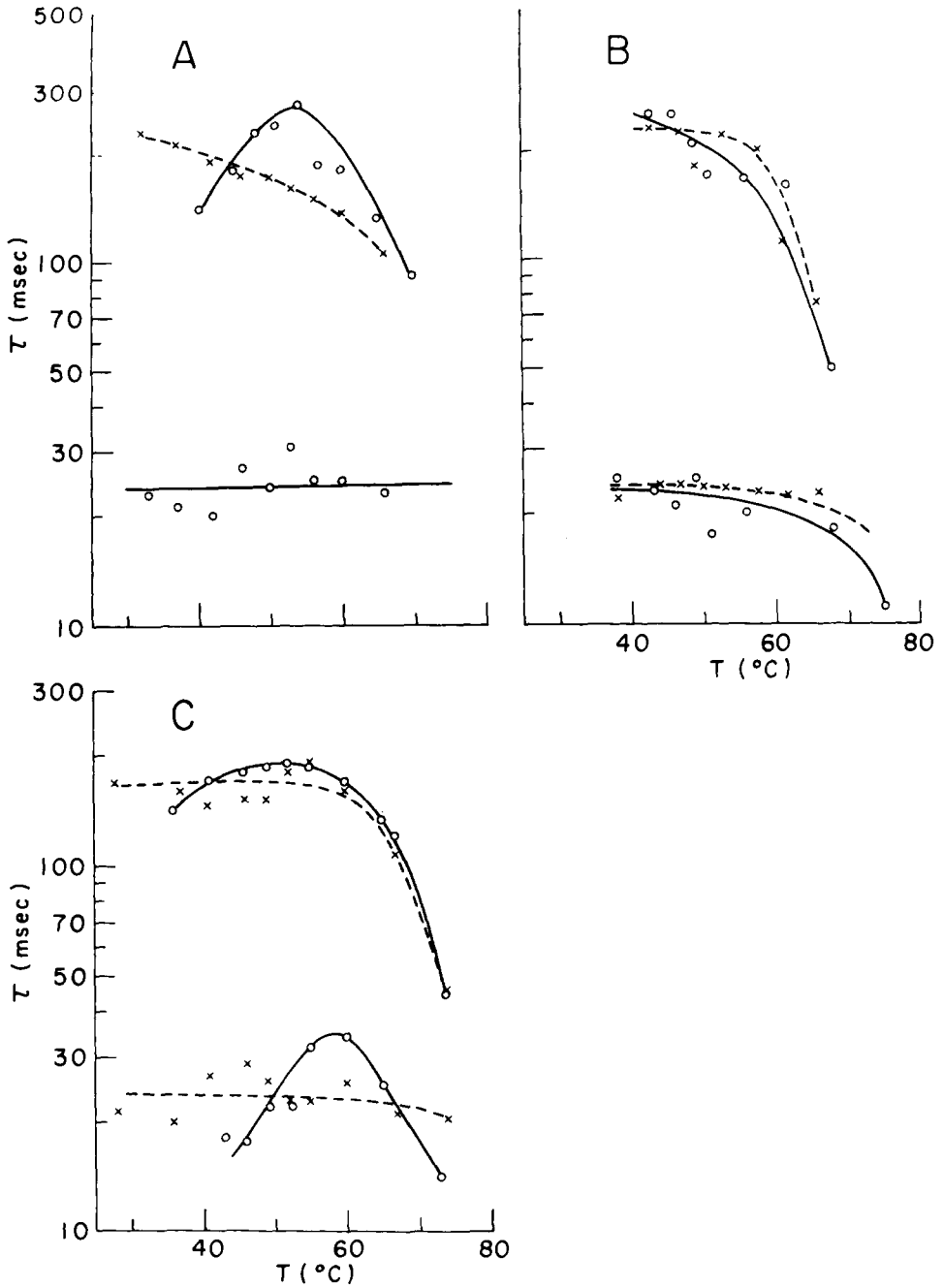

FIGURE 6: Variation of the relaxation times with temperature. In each case the slow relaxation is in the 200-ms and the fast relaxation in the 20-ms time range. (A) RNA-3A; (B) RNA-1; (C) recombined RNA. The open circles (O) are RNA alone and the crosses (X) are RNA + L24. In frame A, the fast τ is only observed for RNA + L24 and is indicated by an open circle (O). Binding buffer was used throughout.

surprisingly small. For each of the RNA fragments alone there is a small red shift in the positive 265-nm band of the CD spectrum when L24 is added. It has been shown that separation of paired bases through the breaking of hydrogen bonds causes a red shift in this CD band in nucleic acid molecules (Hashizume and Imahori, 1967). By this interpretation, L24 induces a decrease in the amount of secondary structure in the separate RNA fragments. In the recombined RNA, however, there is no apparent wavelength shift in the same band suggesting that it requires no major net rearrangements of base pairing to accept the binding of L24. Therefore the interaction of the two fragment RNAs, neither of which is a complete protein binding site alone, promotes the formation of the correct binding site for L24, which must span both RNA molecules since the entire complex is resistant to nuclease digestion.

Just as the position of the CD bands provides information on the base pairing in RNA, the intensities are related to the amount of base stacking (Hashizume and Imahori, 1967). The 265-nm CD band will generally decrease in molar ellipticity with decreasing amounts of stacking. There is no ellipticity change upon addition of L24 to either of the isolated RNA fragments, but there is an ellipticity decrease when L24 is added to the recombined RNA. This same phenomenon is observed when total 30S or 50S ribosomal proteins are added to whole 16S or 23S RNA (Tinoco and Cantor, 1970) and thus addition of the protein(s) alters the arrangement of the base stacking in both rRNA and the isolated L24 binding site.

Heat activation of the recombined RNAs also produces an apparent increase in the amount of base stacking (as evidenced by the increased intensity of the 265-nm band) but no changes

TABLE II: Thermodynamic and Kinetic Properties of the L24-RNA Interaction.

	RNA Alone					RNA + L24				
Fast τ	T_m[d] (deg)	k[a] (s^{-1})	$E_+^{\ddagger}$[e] (kcal/mol)	$E_-^{\ddagger}$[e] (kcal/mol)	ΔH (kcal/mol)	T_m (deg)	k (s^{-1})	$E_+^{\ddagger}$[e] (kcal/mol)	$E_-^{\ddagger}$[e] (kcal/mol)	ΔH (kcal/mol)
RNA-1	61.5	25	+22	−14	−36	61.5	23	+19	−25	−44
RNA-3A	[b]					50.0	[c]			
Recombined RNA	56.5	14	+22	−14	−36	49.0	22	+7	−10	−17
Slow τ										
RNA-1	61.5	5	+48	0	−48	57.5	4	+59	−18	−77
RNA-3A[b]	59.5	2	+37	0	−37	53.0	[c]			
Recombined RNA	62.5	3	+40	−16	−56	55.5	3	+24	−20	−44

[a] k is the rate constant at T_m where $k_i = k_{-i}$. [b] RNA-3A alone showed no detectable fast relaxation time. Thus the parameters for the slow relaxation are calculated assuming the two-state (A $\rightleftarrows$ B) model (Gralla and Crothers, 1973b). [c] The scatter in the experimental data for the relaxation times of RNA-3A plus L24, especially the fast relaxation (see Figure 6a), is sufficiently great that we have not done the calculations. [d] Defined as the temperature at the maximum of the differential melting curve (Figure 5). [e] $E_+^{\ddagger}$ is the activation energy for the reaction in the forward direction (eq 2) and $E_-^{\ddagger}$ is the activation energy for the reaction in the reverse direction.

in the hydrogen-bonded base pairs. In the presence of L24 though, heat activation of the recombined RNAs produces very slight, if any, changes in the CD spectrum indicating that the protein itself is capable of rearranging the RNA binding site into the correct structure for efficient binding, regardless of a heating step. This is unlike the situation of intact 23S RNA and L24 (Schulte et al., 1974) where heating of the mixture is required to stimulate binding of the protein to the RNA. It is possible that the bulk of the 23S RNA which is attached to the L24 binding site prevents the site from rearranging into the proper structure for binding without input of thermal energy.

To summarize our conclusions to this point then, the recombined RNA site exists as an interacting complex between the two subfragment RNAs. Addition of L24 produces no apparent change in the secondary structure (base pairing) but does provoke a small conformational change tentatively ascribed to unstacking of bases. This correlates with the finding (Schulte et al., 1974) that L24 also produces a conformation change in 23S RNA upon binding. The two separate fragments (RNA-1 and RNA-3A) show changes in their secondary structure but not in stacking when binding to the protein (this latter observation may not be functionally important since neither RNA fragment alone comprises the total binding site).

RNA Melting. The thermal transitions of the separate and recombined RNAs, with and without L24, are much broader than observed for tRNA under these ionic conditions, 10 mM Mg^{2+} (Cole et al., 1972), and the hypochromism is also significantly lower than for tRNA (17 vs. 20%). In general the amount of hypochromism is related to the amount of secondary structure present and thus the L24 binding site apparently has less secondary structure than tRNA and, indeed, less than expected simply on the basis of random sequences (Gralla and DeLisi, 1974).

A further pronounced contrast with tRNA is revealed by the kinetic experiments. With or without Mg^{2+} we could not find signals corresponding in time scale with values expected for melting hairpin helices (10 μs to 1 ms; Gralla and Crothers, 1973a; Crothers et al., 1974). The resolvable relaxations are in the time range (10–500 ms) which we have associated with tertiary structure melting in tRNA (Crothers et al., 1974; Yang and Crothers, 1972; Bina-Stein and Crothers, 1975). Hairpin helices may be present in the L24 binding site RNA, but their unfolding is coupled to tertiary interactions of much slower reaction time. The tertiary structure is also different from that in tRNA in that the intramolecular interactions are much less sensitive to Mg^{2+} concentration.

Addition of L24 protein to the RNA fragments results in an increase in average T_m in a static experiment. The temperature-jump melting curves show that this is the resultant of a small *decrease* in the T_m of the two resolved relaxation effects, and an *increase* in the T_m of the unmeasurably fast relaxation arising primarily from unstacking of bases in less ordered regions of the molecule. Hence we conclude that the L24 protein does not appreciably stabilize the ordered regions but binds instead primarily to the less ordered parts, stabilizing them in the process against unstacking of the bases.

Inspection of Table II reveals that the protein does cause significant alterations in the RNA structure as manifested by changes in the energetic and kinetic properties. It is important to note that these kinetic differences are apparent even at low temperatures (Figures 5 and 6) and are not just simply a result of an altered T_m. Furthermore, the relaxation times and amplitudes are reproducible over several temperature jumps at any given temperature, indicating that there is probably no slow, irreversible protein denaturation occurring.

Nuclease and Protease Resistance. There are at least two likely ways in which the interaction of a small protein like L24 could confer nuclease resistance to a physically much larger piece of RNA. One possibility is that the protein is highly extended and contacts nuclease sensitive sites on the RNA. Another is that the RNA is compactly folded and that the single-stranded sites which would otherwise be sensitive to ribonuclease are folded into a small enough region of the molecule to be accessible to direct binding and hence protection by L24. For several reasons the most plausible model is one which includes both of the above hypotheses. Direct electron microscopic visualization of specific antibody binding (reviewed in Lake et al., 1974a) has shown that certain ribosomal proteins have multiple mapping sites on the ribosome surface and are quite extended in conformation. Presently available information concerns only the small subunit, but it is particularly interesting that the best example of an extended protein is S4 (Lake et al., 1974b). This ribosomal protein is very similar to L24 in that its binding site consists of multiple RNA fragments at the 5′ end of the intact RNA, just as in the L24 case. By a direct structural analogy then it seems reasonable that

the L24–23S RNA interaction will be similar to the S4–16S RNA interaction; that is the protein is rather extended and contacts the RNA at several key points which make it less susceptible to nuclease.

The protein L24, too, is highly resistant to enzymatic (trypsin) hydrolysis in the presence of its RNA site, whereas in the absence of RNA L24 is completely digested by trypsin (Branlant et al., 1973; and our own observations). S4 on the other hand, even in the presence of its RNA site, is partially hydrolyzed by trypsin (Schulte et al., 1975). Since L24 does not introduce *major* structural changes in the RNA, it cannot be protected from protease action by burying itself in the RNA structure (which would necessarily involve large structural rearrangements) so as to be completely occluded from enzyme in the bulk phase. Thus L24 is likely not as fully an extended polypeptide chain as S4, just rather more extended than a globular protein of the same size.

The RNA too must be a fairly compact structure. It seems likely that the protein interacts with several foci on the RNA, presumably at nuclease sensitive single-stranded regions. In order for widely separated regions in the RNA primary structure to all be accessible to the smaller (albeit extended) protein and thus be protected by its presence, the RNA must be folded into a compact tertiary structure—the very structure which we see unfolding in the relaxation experiments. This occurs in the recombined RNA site itself which even alone is relatively resistant to ribonuclease. Addition of L24 does not provoke major structural rearrangements but rather subtle conformational changes that provide the additional nuclease protection necessary for high resistance as well as secluding protease sensitive regions of the L24 from attack. As emphasized throughout, this model is similar to the one proposed for the S4–RNA case based on entirely different experimental approaches.

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