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Protein–Ribonucleic Acid Interactions in *Escherichia coli* Ribosomes. Solution Studies on S4–16S Ribonucleic Acid and L24–23S Ribonucleic Acid Binding†

C. Schulte, C. A. Morrison, and R. A. Garrett*

ABSTRACT: A study was made of the interaction of ribosomal proteins S4 and L24 with 16S and 23S RNAs, respectively, of *Escherichia coli*. The optimal temperature and solution conditions of binding were compared and shown to be almost identical. They were heating at 35–45°, 10^{-2}–10^{-1} M Mg^{2+}, 0–0.4 M K^{+}, and pH 7.4–7.9. A critical level of magnesium, associated with a structural change in both RNAs, was required for binding. The RNA and protein binding sites were both shown to be very stable at optimal ionic conditions over a wide temperature range. Only a slight decrease in binding affinity occurred between 0 and 42–48°. Kinetic and sedimentation evidence indicated that under optimal binding conditions conformational changes in a least a fraction of both 16S and 23S RNA populations occurred.

Several ribosomal proteins bind directly to 16S RNA (Mizushima and Nomura, 1970; Schaup *et al.*, 1970a, 1971a; Garrett *et al.*, 1971; Zimmermann *et al.*, 1972) and 23S RNA (Stöffler *et al.*, 1971a,b). Currently the nucleotide sequences of the protein binding sites on both 16S RNA (Schaup *et al.*, 1971b; Zimmermann *et al.*, 1972, 1974) and 23S RNA (Branlant *et al.*, 1973) and the amino acid sequences of many of the RNA-binding proteins are being determined (Wittmann-Liebold, 1971; Reinbolt and Schiltz, 1973). Very little is yet known, however, about either the structural chemistry or the mechanism of the protein–RNA interactions, except for some preliminary work on the latter for the S8–16S RNA interaction (Schulte and Garrett, 1972).

Two proteins, namely S4 and L24, were selected for detailed investigation. S4 binds specifically to 16S RNA (Mizushima and Nomura, 1970) and L24 binds to 23S RNA (Stöffler *et al.*, 1971a,b). For both proteins the nucleotide sequences of the RNA binding sites, which are protected against nuclease digestion by the protein, are almost completely known. They occur at the 5′ ends of their respective RNA molecules and are both about 400 nucleotides long (Schaup *et al.*, 1971b; Zimmermann *et al.*, 1972, 1974; Nanninga *et al.*, 1972; Branlant *et al.*, 1973). Moreover, the amino acid sequence of protein S4 is known (Reinbolt and Schiltz, 1973) and that of L24 is almost completed (R. R. Crichton and B. Wittmann-Liebold, unpublished work).

The results presented here are an attempt to elucidate the factors that influence the specificity of protein–RNA interactions with a view, later, to correlate and coordinate them with the structures of the RNA and protein binding sites. First, the binding conditions of the two proteins were characterized and compared. It was shown that these conditions were almost identical for the two proteins. Second, the stability of the protein and RNA binding sites was examined. They were both shown to be stable over a wide temperature range. Third, the possible occurrence of structural changes in the RNA and proteins was investigated and evidence for conformational changes occurring in the RNAs prior to binding was found.

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Materials and Methods

Proteins. S4 and L24 were extracted from 30S and 50S subunits, respectively, by the methods of Hindennach *et al.* (1971a,b). These were checked for purity by two-dimensional gel electrophoresis (Kaltschmidt and Wittmann, 1970); no contaminating proteins were detected and it was concluded that the proteins were at least 95% pure. They were run in 1-D gels (Leboy *et al.*, 1964) to check for the presence of aggregates; none were detected. Molecular weights of 26,500 and 17,000 were used for S4 and L24, respectively (Dzionara *et al.*, 1970).

S4–16S RNA and L24–23S RNA Complex Formation. A mixture of 16S + 23S RNA was extracted directly from cells (Robinson and Wade, 1968) and the pure RNAs were isolated from subunits (Kurland, 1960). Under standard conditions, protein and a mixture of RNAs were incubated at a 5:1 molar excess of protein in TMK buffer (0.03 M Tris-HCl (pH 7.4), 0.35 M KCl, 0.02 M $MgCl_2$, and 0.006 M 2-mercaptoethanol) at 42° for 1 hr. Nonbound proteins were separated on agarose columns at 2° (Garrett *et al.*, 1971). The complexes were precipitated with 1.5 vol of ethanol for 36–48 hr at −35°. Some complexes, prepared under nonstandard conditions, were dialyzed against TMA 1 buffer (0.01 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.03 M NH_4Cl, and 0.006 M 2-mercaptoethanol) before precipitating with ethanol. The amount of protein bound was estimated quantitatively by an electrophoretic method (Garrett *et al.*, 1971), in which the complex was electrophoresed in 2.25% polyacrylamide gels containing 0.0375 M Tris-HCl (pH 7.3). The protein was specifically stained with Coomassie Brilliant Blue and the band intensity determined with a Joyce-Loebl microdensitometer. The quantitative protein:RNA molar ratio in the complex was estimated approximately (±20%) by comparing the intensity of the stained protein band with calibration curves (Garrett *et al.*, 1971).

Separation of Nonbound Protein on Heated Agarose Columns. In some experiments agarose columns were incubated during the complex preparation in order to separate nonbound protein at different temperatures up to 55°. Little data are available about the temperature stability of agarose except reports from the Sepharose manufacturers that it is unstable above 40°. Therefore, the columns were run first at lower temperatures and then at higher temperatures. After the high-temperature runs, the separating capacity of the column was checked with a mixture of 5 A_{260} units of 16S and 23S RNA and 5 A_{260} units of crude tRNA. At both 4 and 50° the small and large RNAs were completely separated and it was concluded that any damage that may have been incurred by the agarose beads at the high temperatures did not impair their separating capacity.

Analytical Ultracentrifugation. Sedimentation runs were performed in a Beckmann Model E analytical ultracentrifuge using a five-place An-G titanium rotor. Runs were made in double-sector Kel-F cells using ultraviolet absorption optics coupled to a multiplexor accessory, photoelectric scanner, and Dynograph pen recorder. Except where otherwise stated in the text, samples of 1 A_{260} unit of RNA were sedimented at 40,000 rpm and 7°. Although all sedimentaion values were corrected to 20°, RNA concentration and salt (0.35 M KCl) corrections were considered negligible (Cammack *et al.*, 1970; Svedberg and Pedersen, 1959). The degree of heterogeneity of the sedimenting RNA was estimated by measuring the second moment of the integrated sedimenting boundary, as described by Schachman (1957).

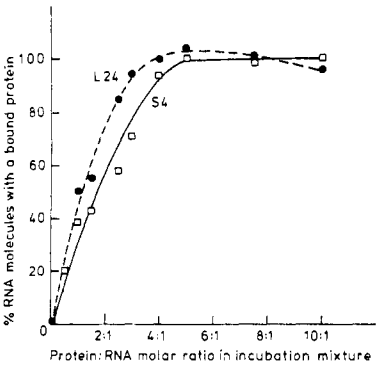

FIGURE 1: Binding curves of S4–16S RNA (□—□) and L24–23S RNA (●- -●) showing saturation. Complexes were prepared under standard conditions and the protein: RNA molar ratio in the incubation mixture was varied over the range 0.5:1 to 10:1. The RNA concentration was constant.

RNA Melting. RNA (0.5 A_{260} unit) in 0.03 M Tris-KCl (pH 7.4), 0.35 M KCl, and $MgCl_2$ in the concentration range 0.001–0.02 M was degassed in sterilized quartz cuvets. Melting profiles were recorded in a Cary 16 spectrophotometer at 255 nm using an interface accessory and Lab/test recorder (Honeywell). Samples were heated continuously in a block by a circulating water bath (Haacke, Berlin) connected to a time-programmed control unit. The sample temperature was recorded directly by a microthermistor.

Results

Specificity of Binding. The capacity of the batches of S4 and L24 to bind specifically to pure 16S and 23S RNA was checked. In addition to establishing that S4 bound to 16S RNA and not 23S RNA, and that L24 bound only to 23S RNA, the proteins were tested for saturation of binding. Complexes were made at increasing protein:RNA molar ratios of mixing. Saturation occurred for both complexes (Figure 1) which indicated that the binding was specific. It was calculated that approximately 1:1 molar binding occurred at the saturation plateau of both proteins (Figure 1).

Ionic Strength Dependence. (a) Mg^{2+}. The degree of binding of S4 and L24 was estimated over the concentration range 0–10^{-1} M Mg^{2+}. The extent of binding was very similar for both complexes over the whole Mg^{2+} range (Figure 2), and a marked binding dependence was detected in the range 10^{-3}–10^{-2} M. At low Mg^{2+} concentrations ($\lesssim 7 \times 10^{-3}$ M), all com-

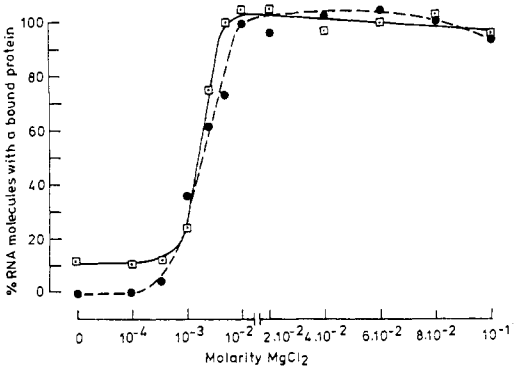

FIGURE 2: The effect of Mg^{2+} on S4–16S RNA (⊡-⊡) and L24–23S RNA (●- -●) binding. Complexes were prepared under standard conditions except that the Mg^{2+} concentration of the TMK buffer was varied. The pH was adjusted to that of the TMK buffer (pH 7.8).

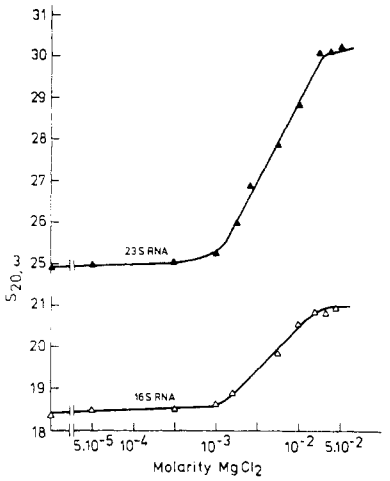

FIGURE 3: Sedimentation coefficients of the 16S and 23S RNA determined in 0.03 M Tris-HCl (pH 7.4) and 0.35 M KCl at increasing Mg^{2+} concentrations. The RNAs were dissolved in this buffer at a concentration of 0.8 A_{260} unit/ml. The Mg^{2+} concentration in the RNA solution was then equilibrated by dialysis for 6–8 hr against the same buffer. The solutions were centrifuged at 40,000 rpm and 7°.

plexes, but especially those of S4–16S RNA, were partly degraded during the incubation, and it was necessary for formation of the S4 complex to limit the incubation at and below 10^{-3} M to 10 min and at 4×10^{-3} M and 7×10^{-3} M to 30 min. Control experiments indicated that this shortening of the incubation had only a small effect on the degree of binding at high Mg^{2+} concentrations. In this low concentration range, S4 binding was detected on both RNAs and the binding was assumed to be nonspecific. As the Mg^{2+} concentration increased, the ratio of nonspecific to specific binding decreased until it reached a minimum at 10^{-2} M Mg^{2+}. Slight nonspecific binding of L24 was also observed between 10^{-3} and 10^{-2} M Mg^{2+}.

In order to test whether the large increase in binding between 10^{-3} and 10^{-2} M Mg^{2+} could be attributed, at least in part, to some change in the RNA structure, the RNA was sedimented at different Mg^{2+} concentrations in an analytical ultracentrifuge. This study was undertaken because it was shown earlier that the sedimentation coefficient of ribosomal RNA is particularly dependent on the Mg^{2+} concentration (Cammack *et al.*, 1970).

In the absence of magnesium, the 16S and 23S RNAs sedimented at 18.5 and 25 S, respectively, in precise agreement with earlier determinations (Cammack *et al.*, 1970). A marked increase in the sedimentation coefficient occurred above 10^{-3} M Mg^{2+} for the 16S RNA, and above 5×10^{-4} M Mg^{2+} for the 23S RNA (Figure 3). At 3×10^{-2} M Mg^{2+} the sedimentation coefficients of 16S and 23S RNAs had increased by 2 and 5 S, respectively. Above 3×10^{-2} M Mg^{2+} both RNAs were highly aggregated. A comparison of Figures 2 and 3 shows that the increases in protein and RNA binding and in the sedimentation coefficients occur over the same Mg^{2+} range. This suggests that there is some correlation between increased binding and a contraction in the RNA structure.

RNA melting profiles were obtained for both RNAs within this critical magnesium range in order to establish whether the binding and sedimentation changes could be attributed to extensive melting of base pairs. The temperature at which the first melting occurred was measured at 10^{-3}, 5×10^{-3}, and 10^{-2} M Mg^{2+}. The observed values were 44, 46, and 48°, respectively. It was concluded that incubation at, or below, 42° did not result in any extensive melting of base-paired regions.

(b) K^+. The dependence of S4 and L24 binding on K^+ was investigated over the salt range 0–2.0 M KCl. Maximum binding occurred for both complexes over a wide salt range, namely 0.0–0.4 M KCl. At higher salt concentrations there was a gradual decrease in binding up to 2.0 M KCl (Figure 4). The percentage binding of L24 was then slightly higher than that of S4.

pH Dependence. The binding of S4–16S RNA and L24–23S RNA was estimated over the pH range 5–8.9. The binding curves for the two complexes are almost identical (Figure 5) with maximum binding occurring in the pH range 7.4–7.9. Slight precipitation of the complexes was observed up to and including pH 7.0. Some hydrolysis of the RNA [35 (±5) %] at pH 8.9 was detected on pyronin G stained RNA gels of the L24–23S RNA complex. The binding at this pH (Figure 5) is corrected for this. At higher pH values considerable RNA hydrolysis occurred.

The sedimentation characteristics of both RNAs were examined over this pH range by analytical ultracentrifugation. No reproducible change in either the sedimentation coefficient or the degree of aggregation of the RNA was observed.

Temperature Dependence. The binding of S4 and L24 was investigated over the temperature range 0–60°. Curves showing the dependence of binding on the incubation temperature are shown in Figure 6. They correspond closely over the

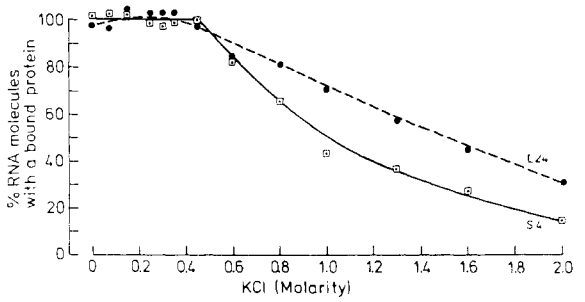

FIGURE 4: The effect of K^+ on S4–16S RNA (⊡–⊡) and L24–23S RNA (●- - -●) binding. Complexes were prepared under standard conditions at increasing K^+ concentrations in the TMK buffer. The pH of the solutions was adjusted to 7.8. Complexes prepared at 0.4 M and higher KCl concentrations were dialyzed for 5 hr against TMA 1 buffer before precipitation with ethanol in order to prevent salt coprecipitation with the RNA–protein complex.

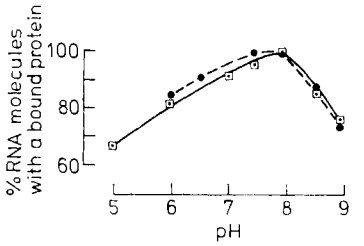

FIGURE 5: The effect of pH on S4–16S RNA (⊡—⊡) and L24–23S RNA (●- -●) binding. Complexes were prepared under standard conditions except that 0.01 M potassium phosphate buffer was used over the pH range 5–7, and 0.01 M Tris-HCl buffer was used over the range 7.4–8.9. The final pH of the buffer, corrected to 25°, is given after addition of KCl, $MgCl_2$, and 2-mercaptoethanol. Complexes prepared outside the pH range 7–8 were dialyzed for 8 hr against TMA 1 buffer, after separation of nonbound protein on agarose columns and before precipitation. This treatment enhanced the solubility of the complexes after precipitation with ethanol. In spite of this treatment the L24–23S RNA complex prepared at pH 5 was insoluble.

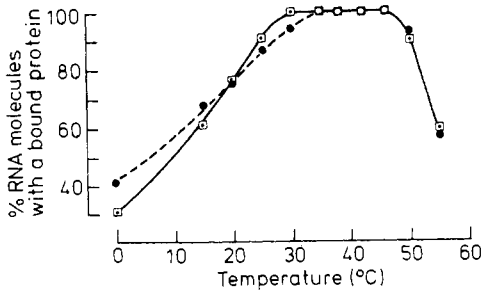

FIGURE 6: The effect of temperature on the amount of S4–16S RNA (⊡–⊡) and L24–23S RNA (●- -●) binding. Complexes were prepared under standard conditions and incubated at different temperatures before cooling in ice and separating nonbound protein. Slight hydrolysis of the 16S RNA occurred at 55°. This point was corrected by calculating the number of protein molecules bound per undegraded 16S RNA molecule.

temperature range 0–55°. At 0°, 32% binding occurred for S4 and 38% for L24 and this increased to maximum binding between 35 and 45°. At 55°, 30% of the 16S RNA, only, was degraded. A correction was made for this (Figure 6). At 60°, at least 50% RNA degradation of both complexes occurred.

Thermal Stability of the Protein and RNA Binding Sites. The actual temperature at which binding occurred, for both proteins, was established by the following experiment. S4–16S RNA and L24–23S RNA mixtures were incubated at 42° for 45 min to ensure that any protein (Schulte and Garrett, 1972) or RNA conformational changes necessary for binding had occurred. The incubation mixture was maintained at a given temperature, between 0 and 55°, for a further 15 min. The complex was separated from nonbound protein on an agarose column thermostated at this temperature and then precipitated with 1.5 vol of ethanol. The extent of binding was then determined. Binding curves for both complexes are very similar (Figure 7). Maximum binding was observed for both proteins between 10 and 20°. This decreased slightly to 80% at 42°. At 48°, a temperature at which melting of the RNA base-paired regions begins, under these buffer conditions, 71% of maximum binding occurred for S4. Extensive 23S RNA hydrolysis occurred at this temperature and no estimate was made of L24 binding. Very slight precipitation of the complex was observed also after the incubation. Considerable hydrolysis of both 16S and 23S RNAs occurred during the incubation at 55°.

In the earlier report on the S8–16S interaction (Schulte and Garrett, 1972) only part of such a curve was given. This was sufficient to show, however, that at 42° only very weak S8–16S RNA binding occurs. Because of the large difference from the S4 and L24–RNA complexes a full curve was prepared and is now included (Figure 7). The form of the curve is different from the S4 and L24 binding curves (Figure 7) in that strong binding only occurred at lower temperatures.

Conformational Study on the Protein and RNA. In order to establish whether heating was necessary to produce a protein or an RNA conformational change, the following experiments were performed on the two complexes. In experiment one each RNA was incubated alone at 42°, under standard conditions, and frozen at −50° within 1 min. On thawing the corresponding proteins were added at 0° and mixed, and the solutions were loaded immediately onto an agarose column at 2°. In the second experiment, the protein solutions were incubated alone at 42°, under standard conditions, frozen at −50°, and thawed. The RNAs were mixed at 0°, and the solutions were immediately applied to an agarose column at 2°.

TABLE I

Sample	Incubation Conditions	S4–16S RNA Binding (%)	L24–23S RNA Binding (%)
1	RNA alone at 42° for 1 hr	66 (63, 67, 69)	83 (75, 79, 87)
2	Protein alone at 42° for 1 hr	9 (7.5, 10)	41 (26, 40, 56)
3	Control complex under standard conditions at 0°	32	38
4	Control complex under standard conditions at 42°	100	100

[a] Complexes were prepared in TMK buffer. The extent of protein–RNA binding was estimated by the standard method. Each complex was prepared in triplicate; individual results are given in parentheses.

The extent of binding for each complex was compared with control complexes prepared at 0 and 42°.

Preincubation of the RNAs produced a marked increase in the binding of both proteins (Table I); a slightly higher increase in L24 binding was observed than for S4. This strongly suggests that a conformational change has occurred in at least a fraction of both rRNA populations. On the other hand, preincubation of the proteins produced no significant increase in binding for either protein; in fact, for S4 there was a decrease probably due to aggregation or reversible denaturation. The latter explanation is supported by the fact that when the RNA was added to the preincubated protein

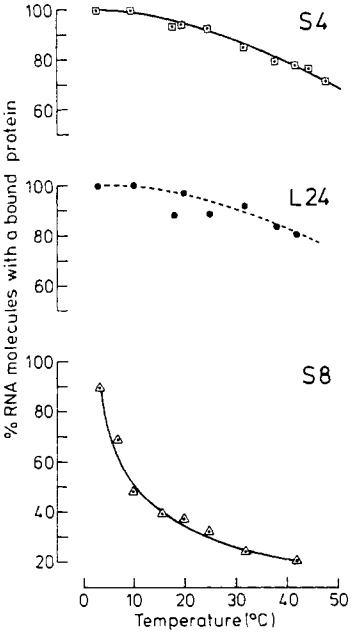

FIGURE 7: The stability of the S4–16S RNA (⊡–⊡), L24–23S RNA (●- -●), and S8–16S RNA (△–△) binding sites at different temperatures. Complexes were prepared under standard conditions but they were incubated for 45 min at 42°. The incubation temperature was then reduced to the given temperature for a further 15 min before separating nonbound protein on an agarose column thermostated at the latter temperature. After the run the complex was cooled and precipitated with ethanol.

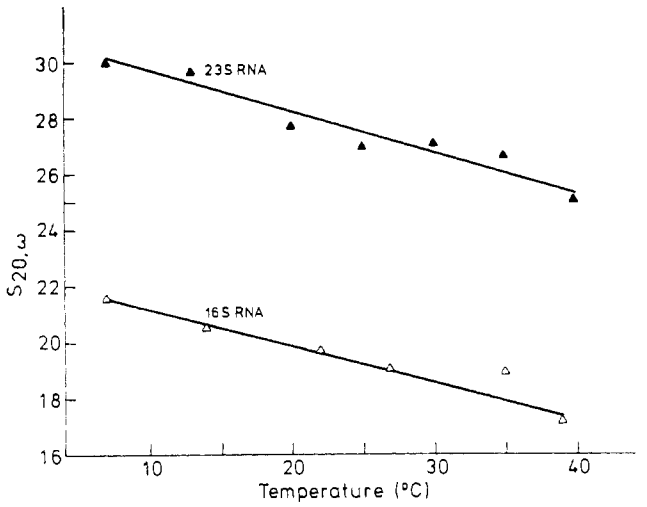

FIGURE 8: Pure RNA solutions at a concentration of 1.0 A_{260} unit/ml of TMK buffer were equilibrated for 40 min at temperatures in the range 7–40° in a preheated AN-G rotor. They were ultracentrifuged in the standard way. Runs were made in duplicate and the sedimentation coefficients were corrected for temperature and averaged

solution, 3 min before freezing, 72 and 82% binding occurred for S4 and L24, respectively.

The heat-induced structural change in both RNAs was investigated further by ultracentrifuging at increasing temperatures. Three parameters were measured: (1) the sedimentation coefficients of the RNAs, (2) the conformational homogeneity as represented by their second moments (Schachman, 1957), and (3) the degree of aggregation of the RNAs. A significant decrease in the sedimentation coefficient of both 16S and 23S RNAs occurred over the range 7–40° (Figure 8). This suggested that some gradual opening of the structure had occurred which could have resulted in increased accessibility to the protein binding sites. The second moment also showed a decrease over this temperature range (Figure 9a), a result which suggested that the RNA became more conformationally homogeneous at higher temperatures. Since the sedimentation coefficients of both RNAs show a very small concentration dependence, in the concentration range used (Cammack *et al.*, 1970), this decrease in second moment could not be attributed to a concentration effect (Schachman, 1957). There was also a gradual decrease in the degree of aggregation of the RNAs with increasing temperatures, especially of 23S RNA, which could contribute to, but not wholly account for, the increased protein–RNA binding with temperature.

The possible occurrence of a significant RNA conformational change resulting from the interaction of the proteins with the RNAs was also investigated by measuring the sedimentation coefficients of the two complexes and their respective RNAs. All runs were made at 39 and at 7° after cooling in TMK buffer. No significant differences outside the error limits of ±0.5 S were observed. At 39°, 17 S was obtained for the 16S RNA and complex, and 25 S was estimated for the 23S RNA and complex. At 7°, 21.5 S was obtained for the 16S RNA and complex, and 30 S for the 23S RNA and complex.

Discussion

Some of the solution conditions which are essential for *in vitro* 30S subunit reconstitution (Traub and Nomura, 1969) are relatively uncritical for the two protein–RNA interactions investigated. Maximum specific binding occurs over a wide

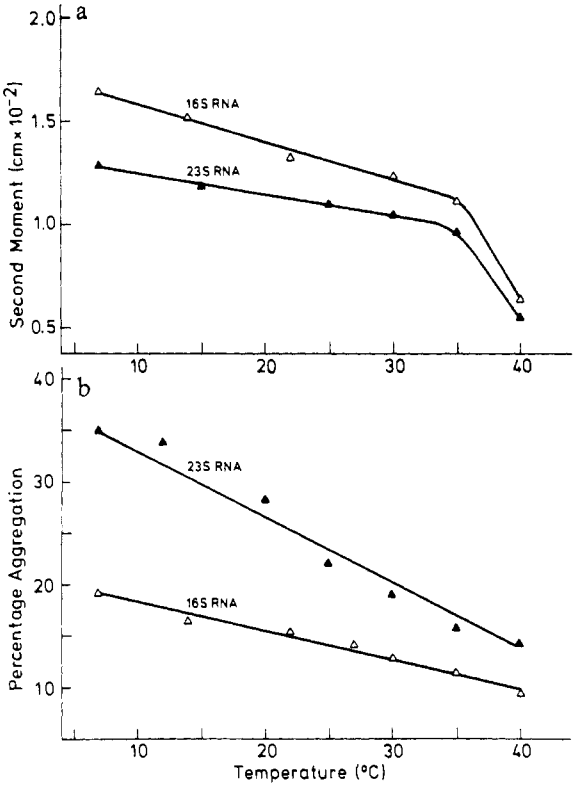

FIGURE 9: (a) The second moment of the integrated sedimenting boundary is plotted as a function of temperature. (b) The percentage of RNA aggregation is given as a function of temperature. In both a and b △ represents 16S RNA and ▲ represents 23S RNA.

K^+ range and unlike S8–16S RNA (Schulte and Garrett, 1972), no significant nonspecific binding occurred at low K^+ concentrations. Also there was a broad pH optimum. The dependence on Mg^{2+} was different, however. Clearly, Mg^{2+} is essential for a stable protein–RNA interaction. The most likely explanation of the binding and sedimentation results is that magnesium ions contract and stabilize the RNA structure in the range 10^{-3}–10^{-2} M Mg^{2+}, the minimum range of magnesium required for specific protein binding. Since the RNA melting data render it unlikely that large numbers of base pairs are disrupted in this structural change, it is likely that some packing of the RNA structure occurs.

There was a strong dependence of the protein–RNA binding on temperature which could be correlated to some extent with the sedimentation properties of the RNAs. At increasing temperatures of both RNA solutions, there were significant decreases in (1) the sedimentation coefficients, (2) the second moments, and (3) the degree of aggregation. The sedimentation coefficient and second moment data suggest that whereas at low temperatures some RNA conformational heterogeneity occurs, at higher temperatures a more open and homogeneous structure is formed. A similar conclusion has also been reached by A. Muto and R. Zimmermann (personal communication) from other protein–RNA binding experiments. The small decrease in aggregation which occurred at increasing temperatures could also contribute to the increased protein–RNA binding at higher temperatures since the proteins might not bind to RNA aggregates.

There is one important difference between the earlier results for the S8–16S RNA interaction (Schulte and Garrett, 1972) and those presented here for the S4–16S RNA and L24–23S RNA interactions. When S8 (after heating and

cooling) was mixed with 16S RNA at 0° a high level of binding occurred (65% of maximum binding), whereas for S4 and L24 much lower binding occurred (40% or less). This suggests either that the conformation of S8 is more stable at 0° or that the RNA binding site of S8 is more accessible at 0° than the RNA binding sites of S4 and L24. It may be the latter because if the RNA is conformationally heterogeneous at low temperatures, as is suggested by the sedimentation data (Figure 9), the small rigid RNA binding site of S8 (~40 nucleotides) (Zimmermann *et al.*, 1974) is more likely to be available and in the appropriate binding conformation than the highly complex and flexible binding sites of S4 (~500 nucleotides) and L24 (~350 nucleotides) (Schaup *et al.*, 1972; Branlant *et al.*, 1973; Zimmermann *et al.*, 1974).

The study of dependence of binding on temperature revealed another significant difference between the S8–16S RNA interaction, on the one hand, and those of S4–16S RNA and L24–23S RNA on the other. The interaction of S8 is much weaker at higher temperatures than those of S4 and L24 (Figure 7). Although this effect could be due to structural changes in the S8 protein at higher temperatures (and temperature-sensitive mutants of *Escherichia coli* have been isolated with altered S8 proteins; H. G. Wittmann, personal communication), the result could also be interpreted in terms of the instability, at elevated temperatures, of a small weakly base-paired section of the RNA binding site of S8 (Zimmermann *et al.*, 1974). The effect of temperature on the protein structures is currently being investigated (C. A. Morrison and R. A. Garrett, unpublished work).

Sypherd (1971) demonstrated that at 40°, total 30S subunit proteins could alter the structure of heat-denatured 16S RNA. Moreover, Traub and Nomura (1969) claimed that total 30S subunit protein facilitates a conformational change in native 16S RNA at this temperature. Both of these changes were shown to occur after the formation of an intermediate (RI) particle at 0°. It has been demonstrated, at least for the latter intermediate particle (Homann and Nierhaus, 1971; C. Schulte and R. A. Garrett, unpublished work), that at least some S4 protein molecules were already bound before the conformational change occurred. The fact that we found no significant sedimentation coefficient differences between the S4–16S RNA complex and the pure RNA is therefore compatible with these results.

The almost identical binding results for the two complexes have some relevance to the biological significance of the L24–23S RNA interaction in particular, and to 50S subunit protein–23S interactions in general. While 30S subunits can be reconstituted *in vitro* in TMK buffer, 50S subunits have not yet been. The doubt remains, therefore, that the binding of 50S subunit proteins to 23S RNA in TMK buffer has no biological significance. The similarity of the conditions for the specific S4–16S RNA and L24–23S RNA binding, demonstrated here, should finally eliminate this doubt.

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