Volume 7 Number 2 1979 Nucleic Acids Research

Binding of magnesium ions and ethidium bromide: comparison of ribosomes and free ribosomal RNA

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Received 6 July 1979

ABSTRACT

Comparative studies of free ribosomal RNA and ribosomes were made with two probes, Mg^{++} ions and ethidium bromide, which interact with RNA in different ways. Mg^{++}. E.coli 16 S rRNA and 30 S ribosomes were equilibrated with four different buffers. Equilibration required several days at 4° and several hours at 37°. In all buffers ribosomes bound more Mg than free rRNA, the difference sometimes reaching 20-30%. Ribosomes were more resistant than free rRNA to heat denaturation and their denaturation was more highly cooperative. Ribosomes that bound more Mg^{++} had higher denaturation temperatures. Ethidium bromide. Fluorescence enhancement studies of ethidium intercalation showed the free 16 S rRNA to have 50-80 binding sites per molecule. A large fraction of these sites were present and accessible in the ribosome, but their ethidium-binding constants were reduced by an order of magnitude. In addition, free rRNA contained a small number of very strong binding sites that were virtually absent in the ribosomes.

INTRODUCTION

In our investigations of the ribosome, we envisage it as a complex whose flexible conformation is formed and stabilized by the mutual interactions among its components, which include not only RNA and proteins but also specific monovalent and bivalent cations (1). The formation of the ribosomal structure requires that the macromolecular components have appropriate conformations, as shown by the recent demonstration that the method of preparation of ribosomal RNA influences its ability to interact with proteins during ribosome reconstitution (2). Further, it is known that the functional conformation of the total ribosome is maintained by a finely tuned complex of macromolecular interactions in which the cations play a decisive role (1,3-5). Thus, certain changes in the ionic environment which do not change overall structural properties of the ribosome (e.g., ultraviolet absorption spectrum, circular dichroism spectrum) do nevertheless change something in the detailed conformation of component macromolecules, with drastic effects on functional activity (3-5).

The use of specific probes is one of the few methods adequate for studying the conformation of a macromolecule both when it is free and when it is part of a larger complex; and comparison of the two states can yield information on how its conformation is influenced by neighboring components in the complex. The present communication deals with the 16 S rRNA of the E.coli 30 S ribosome. We have employed two characteristic probes for RNA: Mg^{++} ions and ethidium bromide. Mg^{++} ions are known to stabilize the conformation of RNA in solution, and are required for ribosomal activity. Ethidium ions become intercalated into base-paired and stacked regions of nucleic acid (6-8), and the resulting enhancement of ethidium fluorescence is evidence of the presence of such regions. The behavior of free 16 S rRNA and 30 S ribosomes has been compared in four different buffers, and we describe several changes that take place in the behavior of the RNA when it is incorporated into the ribosome.

MATERIALS AND METHODS

Buffers

Buffers are designated as MTK a-b-c. M=magnesium acetate, T=tris or tricine (pH 7.6-7.8), and K=potassium acetate; and a,b and c are their respective millimolar concentrations. MTK 1-10-20 is 1 mM magnesium acetate - 10 mM tricine (or tris) - 20 mM potassium acetate; MTK 1-10-100 is the same but with 100 mM potassium acetate; etc. In certain cases, specified in the text,the buffers also contained 2 mM dithiothreitol or 6 mM β-mercaptoethanol.

Ribosomes and RNA

30 S ribosomal subunits were isolated from E.coli MRE 600 as described previously (9), except that they were sometimes recovered from the preparative sucrose gradient fractions by an overnight high speed centrifugation instead of being precipitated by polyethylene glycol 6000. 16 S RNA was prepared from sodium dodecyl sulfate-treated 30 S ribosomes by phenol extraction (9). Stock solutions of ribosomes and RNA were dialyzed against MTK 1-10-20 and either kept in ice for immediate use or stored in liquid nitrogen. The preparations used in the experiments reported below were shown to be homogenous by electrophoresis in 3% acrylamide - 0.5% agarose gels in buffer MTK 1-10-20 and also, in the case of ribosomes, by analytical ultracentrifugation. Their concentration,expressed as nucleotide concentration, was determined by hydrolyzing duplicate samples of ribosomes or RNA to mononucleotides with 0.4 M NaOH at 37° overnight. The hydrolysate

was diluted with 4 volumes of 1 M sodium phosphate (pH 7.0) and its absorbance measured at 260 nm and 290 nm. The nucleotide concentration (mM) was taken as equal to 0.1015 x ($A_{260\ nm}$ - $A_{290\ nm}$).

Equilibration of ribosomes and RNA with buffers

One ml or less of 16 S RNA or 30 S ribosomes, approximately either 5 or 10 mg/ml in MTK 1-10-20, were dialyzed against about 300 ml of buffer in a glass vessel on a rocking platform shaker. Dialysis was carried out for at least 3 days in a cold room at about 4° or for at least 5 hours at 37°. The outside dialysis fluid was changed 2 or 3 times during the process. When RNA and ribosomes were to be compared in the same buffer, they were dialyzed in the same vessel. Dialysis tubing was boiled in 0.01 M EDTA and in distilled water, and was thoroughly washed with distilled water before use.

Mg^{++} binding

Magnesium concentration was determined with a Perkin-Elmer Atomic Absorption Spectrophotometer, model 306, using a standard solution of $MgCl_2$ whose Mg concentration had been determined by titration with EDTA (10). The same stock standard solution was used in all experiments. In each experiment several appropriate dilutions of the standard solution were assayed to verify that the response of the instrument was linear with Mg concentration in the range employed. Duplicate aliquots of each equilibrated ribosome and RNA solution and of each corresponding outside dialysis fluid were diluted to the same concentration range; and the atomic absorption of each was determined against the standard solution of nearest Mg concentration, read immediately afterward. All dilutions were made with distilled water whose Mg content was assayed and shown to be negligible. Separate duplicate samples were taken for determination of the ribonucleotide concentration.

Thermal denaturation

Stoppered cuvettes with a 10 mm light path and an internal width of 2 mm were used. The internal surface of the cuvettes was coated with silicone preparation (Sigmacote SL-2, Sigma Chemical Co.) to prevent adsorption of ribosomes on the cuvette walls, since appreciable adsorption may take place in cuvettes with a high ratio of surface to volume. Solutions of ribosomes and RNA (absorbance at 260 nm = 0.4 to 0.6) were degassed under vacuum before being put into the cuvettes, which were then closed with tight-fitting silicone rubber stoppers. The experiments were carried out with a recording spectrophotometer (Gilford, model 250) equipped with an automatic cuvette positioner, reference compensator, dual wavelength-

attachment and thermosensor. Temperature was controlled by circulating a water-glycol mixture through the side plates of the cell compartment, using a Haake thermostatted circulating bath and temperature programmer. The cuvettes were allowed to come to temperature equilibrium in the cell compartment, after which the temperature of the compartment was raised at a constant rate of about 50°/hour. Compartment temperature and the absorbance of each solution were recorded in turn, alternately at 260 nm and 320 nm to monitor absorbance and turbidity, respectively. Turbidity appeared in the ribosome solutions at a temperature characteristic of the buffer, and the ribosome melting curves were discontinued at this point. Absorbance values, taken from the recorded curves, were multiplied by the specific volume of water at the same temperature to correct for thermal expansion of the solution.

Ethidium bromide binding

The intercalative binding of ethidium bromide by RNA and ribosomes was determined by the fluorescence enhancement method (7), using a Perkin-Elmer Fluorescence Spectrophotometer, model MPF-44A. The excitation and emission wavelengths (uncorrected) were 550 nm and 610 nm. Intercalation caused an approximately 50-fold enhancement of the fluorescence of ethidium at these wavelengths. The experiments were carried out at room temperature in square cuvettes of 10 mm internal width. Each experiment was begun with 2.5 ml of equilibrated RNA or ribosome solution about 60-100 μM in nucleotides (determined by alkaline hydrolysis, see above). Successive 5- or 10-μl aliquots of ethidium bromide stock solutions of increasing concentration were added with a glass micropipette and the contents of the cuvette were mixed after each addition. Fluorescence intensity was recorded until a stable value was reached, i.e., until binding equilibrium was attained; this usually occured during mixing, Twenty to 30 additions of ethidium bromide were made for each binding curve, and the concentration of ethidium bromide in the cuvette ranged from about 0.005 μM at the start to 30-40 μM at the end. Nucleotide and ethidium concentrations were corrected for dilution by the added aliquots of ethidium bromide.

The concentrations of bound and free ethidium were calculated from the commonly used equation $C_B = (F - \phi_F C_T)/(\phi_B - \phi_F)$, derived from the relationships $F = C_B\phi_B + C_F\phi_F$ and $C_T = C_B + C_F$; where F is the observed fluorescence minus any background fluorescence; C_T, C_B and C_F are the respective μmolar concentrations of total, bound and free ethidium; and ϕ_B and ϕ_F are the fluorescence of 1 μM solutions of bound and free ethidium, respectively. For each buffer, ϕ_F was measured directly with solutions of

ethidium bromide lacking RNA and ribosomes, while ϕ_B was taken as the fluorescence in the presence of RNA at the lowest concentration or concentrations of ethidium employed. The concentration of ethidium bromide stock solutions was determined from their absorbance at 480 nm, assuming a molar extinction coefficient of 5600 (6). Fluorescence was corrected for absorption of the exciting light by ethidium (inner filter effect) according to LePecq and Paoletti (7) using the uncorrected values of C_B and C_F and assuming that the μmolar extinction coefficient of free ethidium at 550 nm is 0.00073 and that of bound ethidium is 0.00244 (6). The corrected value of F was then used to recalculate C_B and C_F.

Other procedures

Sedimentation analyses were carried out in a Beckman Model E analytical ultracentrifuge at 20° and 52,000 revs/min at a ribosome concentration of 2 - 3 mg/ml, using Schlieren optics.

Phenylalanyl-tRNA binding activity was assayed essentially as described before (4) in the presence of saturating concentrations of (^{14}C)Phe-tRNA and polyuridylic acid. The 50-μl assay mixtures contained 8 - 9 μg of ribosomes and were incubated at 0° for 90 min. Ribosomes do not equilibrate with the assay medium under these conditions and their activity is determined by their previous treatment. In the example shown below, 40% of the ribosomes were active in the most active sample.

RESULTS AND DISCUSSION

Equilibration with the ionic medium

Four buffers were employed in this study: MTK 1-10-20, MTK 1-10-100, MTK 7-10-300 and MTK 20-10-300. They were chosen to provide a variety of ionic environments with respect to Mg^{++} ion concentration, Mg/K ratio and ionic strength. The last two buffers are known to support ribosomal activity *in vitro*, and MTK 20-10-300 also supports *in vitro* reconstitution of active 30 S ribosomes (11). MTK 1-10-20 has been used extensively in this laboratory for the preparation of stable ribosome fragments (9, 12, 13).

Kinetic studies were carried out to determine the time required for 30 S ribosomes, originally dissolved in MTK 1-10-20, to come to equilibrium with the other buffers. With continual mixing of the solutions inside and outside the dialysis bag, three days were required at 4° to bring the Mg^{++} concentration inside the bag to its final value. At 37° 3 to 5 hours were required. It was assumed that these conditions would also suffice for free RNA.

It has been pointed out before that the equilibration of nucleic acids and especially ribosomes with Mg^{++} is a slow process (14-16) and the same is probably true of other ions. The time required apparently depends on many factors, such as temperature, stirring, initial and final ion concentrations, macromolecule concentration, etc. The passage of the buffer ions through the dialysis membrane is rapid, equilibrating in about an hour at 4° under our conditions; therefore the time-consuming processes are those that take place inside the ribosome. During this period of changing internal ionic makeup, the ribosome is likely to undergo a series of transient conformational shifts whose effects may be difficult to evaluate and reproduce and which, in some cases, must be avoided. For example, in order to maintain the integrity and stability of ribosome fragments, we have found it essential to keep them in full equilibrium with the medium (9, 12, 13).

Equilibrated ribosomes sedimented as a single symmetrical peak in the analytical ultracentrifuge with sedimentation constants (S_{20}) ranging from 27 S in MTK 7-10-300 to 29 S in MTK 1-10-20. In the absence of a sulfhydryl reducing agent, a minor 40 - 50 S component, probably a dimer, occasionally appeared in MTK 7-10-300 and 20-10-300; it was not seen when dithiothreitol or β-mercaptoethanol were present.

Binding of Mg^{++} ions

After equilibration, the amount of Mg^{++} bound by ribosomes and free RNA was determined as described in Materials and Methods. The results are shown in Table 1. The amount of Mg^{++} bound varied with the buffer, the major correlation being with the Mg/K ratio of the buffer. In general, ribosomes bound more Mg^{++} than did free RNA, the difference sometimes reaching 20-30%.

TABLE 1. BINDING OF Mg^{++} IONS BY 30S RIBOSOMES AND 16S rRNA

		Mg^{++} bound (moles per mole of nucleotide)		
Equilibration temperature	MTK Buffer	Ribosomes	rRNA	Ribs./RNA
4°	1-10-20	0.33±0.0036 (11)	0.31 (2)	1.1
	1-10-100	0.18±0.0035 (4)	0.15 (1)	1.2
	7-10-300	0.16±0.0075 (5)	0.14 (2)	1.1
	20-10-300	0.23±0.0036 (5)	0.19 ± 0.0085(3)	1.2
37°	1-10-20	0.34±0.0020 (8)	0.34 (2)	1.0
	1-10-100	0.19±0.0017 (4)	0.15 ± 0.0028(3)	1.3
	7-10-300	0.21 (2)	0.16 (2)	1.3
	20-10-300	0.26±0.0016 (4)	0.25 (2)	1.0

Results are given as: Mean ± standard error of mean (number of experiments).

Binding was higher at 37° than at 4°.

The competition between monovalent and bivalent cations for binding sites on E.coli ribosomes is well documented (14, 15, 17-21). This competition is regulated by the relative concentrations of the two types of cation rather than by their absolute concentrations. This is demonstrated most clearly in our data (Table 1) by the fact that the highest Mg^{++} binding occurred in MTK 1-10-20, a buffer with the lowest absolute Mg^{++} concentration employed but with a high Mg/K ratio. The influence of the bivalent/monovalent cation balance of the buffer on ribosomal stability and conformational flexibility has been noted by Ghysen et al. (22).

The comparative binding capacity of ribosomes and free rRNA is a matter of considerable interest. Polyamines, like cations in general, compete with and displace Mg^{++} from ribosomes and RNA (16, 21, 23). The predominantly basic ribosomal proteins are polyamines and would be expected to do the same, in which case ribosomes would bind less Mg^{++} than free rRNA in the same buffer. However, this is not the case. Our results show the opposite effect: that ribosomes bind more Mg^{++} than free rRNA, the observed difference sometimes reaching 20 or 30%. Our results are in partial agreement with the earlier reports of Goldberg (14) and Choi and Carr (18), who also showed that ribosomes do not have a diminished Mg^{++}-binding capacity compared with free RNA, but reported the two to have essentially equal capacities.

Since the basic amino acid residues of the proteins do not displace Mg^{++} ions from the RNA phosphate groups, they must either not interact directly with these groups or else do so only at the expense of monovalent cations. Either case imposes rather special requirements on the construction of the ribosome.

There are two possible explanations for our observation that ribosomes bind more Mg^{++} than does free rRNA. One is that additional Mg^{++} is bound by the abundant glutamic and aspartic acid residues (24) of the ribosomal proteins. The other is that the proteins affect the conformation of the RNA in the ribosome in such a way as to enhance its capacity to bind Mg^{++}. Since bivalent Mg^{++} can interact with two phosphate groups (14, 18), the binding affinity of Mg^{++}-binding sites would be influenced by the spatial arrangement of structural elements involving more than one nucleotide. The ribosomal proteins, which play a critical role in forming the tertiary structure of the ribosome, cause the rRNA to fold into a more compact and stable conformation by bringing formerly distant segments of RNA closer together (25). This could well produce a spatial arrangement with a higher affinity for Mg^{++} ions.

Thermal denaturation

Studies comparing thermal denaturation profiles of free rRNA and ribosomes have shown that the RNA gains conformational stability when incorporated into the ribosome. In the nucleoprotein complex the onset of RNA denaturation is deferred to a higher temperature and becomes cooperative, its initiation depending upon the prior disruption of non-covalent interactions in which the proteins participate (25-28). The role of Mg^{++} was not clarified in these experiments since Mg^{++}-binding was not measured and, for technical reasons, most of the buffers employed had unusually low Mg^{++} concentrations and ionic strengths. We have therefore performed thermal denaturation experiments in order to evaluate and compare the influence of the buffer and the bound Mg^{++} on the conformational stability of rRNA and ribosomes.

At room temperature the absorbance at 260 nm of ribosomes and rRNA was the same and was not significantly affected by the equilibration buffer (Table 2), indicating that they have the same amount of secondary structure under these conditions, but not necessarily implying that the structures are identical. Fig. 1. illustrates the thermal denaturation patterns observed when the temperature was raised. The same general pattern was found in each of the four buffers (Fig. 1 a - d). Compared with free rRNA, ribosome denaturation began at a higher temperature but, once started, proceeded more rapidly. That is, the ribosomes were more resistant to heat denaturation and their initial denaturation was more highly cooperative. This effect is clearly due to the proteins.

In addition, however, ribosomal heat stability was markedly affected by bound Mg^{++}. This is seen most clearly in Fig. 1, which compares the heat denaturation curves of ribosomes equilibrated against each of the four buffers. The curves fall into two distinctly separate groups, a low stability group and a high stability group. The buffers conferring high heat-

TABLE 2. ABSORBANCE AT 260 nm

	30 S ribosomes	16 S rRNA
ε (P) 260	7634 ± 23 (32)	7564 ± 33 (21)
Hyperchromic effect	53.5 ± 0.5% (30)	53.1 ± 0.6% (20)

ε (P) 260: Absorbance of a solution 1 M in nucleotides.
Hyperchromic effect: Per cent increase in absorbance after total alkaline hydrolysis to mononucleotides.
Results: Mean ± standard error (number of determinations).
The differences between ribosomes and RNA are not significant.

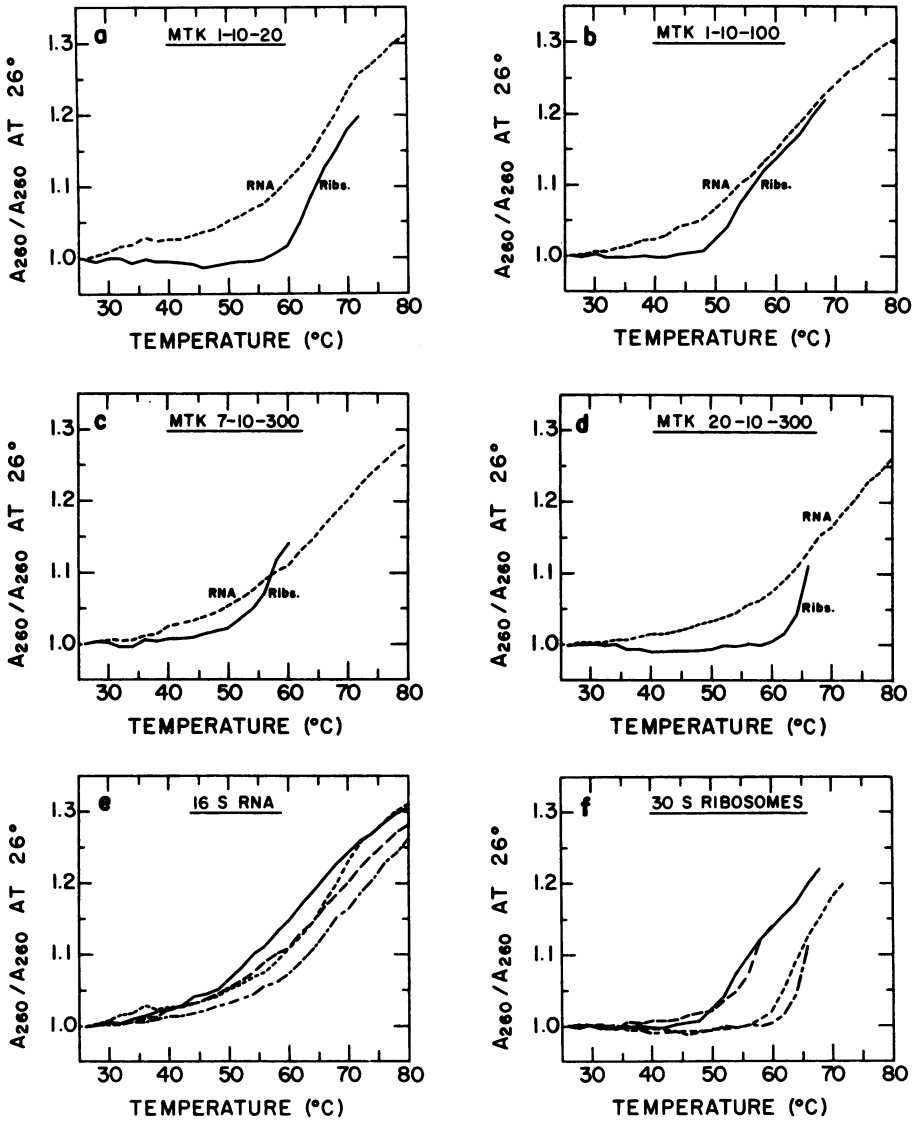

Figure 1. THERMAL DENATURATION PATTERNS

Panels e and f: MTK 1-10-20 _ _ _ _ _; MTK 1-10-100 _____;

MTK 7-10-300 ___ ___ ___ ___ ___; MTK 20-10-300 ___ _ ___ _ ___.

stability were MTK 1-10-20 and MTK 20-10-300, the two buffers in which Mg^{++}-binding was the highest (Table 1). Thus, the heat-stability of the ribosome

is determined not by the external ionic environment but, rather, by the amount of Mg^{++} actually bound by the ribosome. This clear effect was not seen with the free rRNA (Fig. 1 e), where the differences were less striking and appear to have been correlated to a large extent with the Mg^{++} concentration and ionic strength of the medium. Thus, both the ribosomal proteins and the bound Mg^{++} appear to play major (and perhaps mutually reinforcing) roles in stabilizing RNA conformation in the ribosome (see also ref. 29).

Activity

It is known that the ionic composition of the medium is critically important in determining the ability of the ribosome to carry out functional reactions. Ribosomes are inactivated by the withdrawal of specifically required cations (e.g., Mg^{++}, or K^{+} and NH_4^{+}), but can regain activity when the depleted cations are restored; however, activation requires, in addition, a heat treatment, usually about 30 min at 37° or 40° (3-5). Experiments in this laboratory have shown that time can replace heat in the reactivation process. When placed in a buffer that can support activity, inactivated ribosomes slowly regain activity even if kept in the cold, the process now requiring several days (Spitnik-Elson, unpublished). The times required for activation at the different temperatures resemble those required for equilibration with Mg^{++} (see above). We therefore examined the effect of ionic equilibration versus non-equilibration on an activity of the 30 S ribosome, the messenger RNA-directed ability to bind aminoacyl-tRNA.

Table 3 shows the results of a representative experiment; several other experiments gave similar patterns. Ribosomes initially dissolved in MTK 1-10-20 were either allowed to equilibrate for three days in the cold

Table 3. Phe-tRNA BINDING ACTIVITY OF 30S RIBOSOMES
EFFECT OF IONIC EQUILIBRATION

	Activity (cpm per μg ribosomes)					
MTK Buffer	1-10-20		7-10-300		20-10-300	
Pre-treatment	None	Heated	None	Heated	None	Heated
Not equilibrated	-	-	201	208	254	408
Equilibrated	253	251	236	280	334	469

All buffers contained 6 mM β-mercaptoethanol.
Equilibration: 3 days at 4°.
Pretreatment: Heated 30 min at 40°; then chilled to 0°.
Assay in presence of poly-U: 90 min at 0°; see Methods for details.

with MTK 7-10-300 or MTK 20-10-300, or else were diluted into these buffers about an hour before the assay, in which case they did not come to equilibrium with the new buffer. Although the assay medium and conditions were the same for both, the pre-equilibrated ribosomes were more active. When the usual heat treatment, 30 min at 40°, was applied, there was a further increase in activity. It will be recalled that ribosomes in these two buffers bind more Mg^{++} when equilibrated at 37° than at 4° (Table 1). The conformation of the ribosomes equilibrated at 37° therefore not only bound more Mg^{++} than that equilibrated at 4° but also was more active in the assay employed. The 30-minute heat treatment was apparently too short to allow full equilibration at 40° since this requires several hours (see above), and this probably explains why the non-equilibrated ribosomes did not overtake the equilibrated ones during the heat treatment. Ribosomes equilibrated in MTK 1-10-20 were not made more active by the heat treatment (Table 3); neither did they bind more Mg^{++} at 37° than at 4° (Table 1).

In general, the results indicate that activity assays may not express the true capacity for activity of ribosomes in a given medium unless the ribosomes have been allowed to come to equilibrium with the medium. As expected, there was an optimum value for bound Mg^{++}. The most active ribosomes were those equilibrated against MTK 20-10-300, whose bound Mg^{++} was intermediate between the values attained in MTK 1-10-20 and 7-10-300, both of which supported activity, but at a lower level. Although 1 mM Mg^{++} supported activity in MTK 1-10-20, it does not do so in buffers with higher concentrations of monovalent cations. This again shows that it is the bound Mg^{++} and not the external Mg^{++} concentration that is decisive.

The binding of messenger RNA and aminoacyl-tRNA takes place on the surface of the ribosome where equilibration with the medium is presumably rapid. However, the ribosomes did not attain their highest activity rapidly. We believe that the slow limiting process in question is the movement of ions into and out of the ribosome and the consequent conformational changes that take place there.

Ethidium bromide binding

Ethidium ions are known to bind to nucleic acids in two ways: (a) by intercalation, i.e., insertion of the planar molecule between two adjacent stacked base-pairs (6-8) or bases (30,31); or (b) by non-intercalative electrostatic interaction with the phosphate groups of structured or unstructured polynucleotides (6, 7). Only intercalation causes enhancement of ethidium fluorescence (32). Consequently, when its binding is measured by

fluorescence enhancement, ethidium is a specific probe for stacked structure in polynucleotides. In addition, there is evidence that the properties of the intercalation complex vary with the base composition, geometry and flexibility of the intercalation site (31, 33). Thus it may also be possible to use ethidium as an indicator of conformational differences in polynucleotides and nucleoproteins. We therefore examined and compared the intercalative binding of ethidium by free rRNA and 30 S ribosomes, using the fluorescence enhancement technique.

Binding curves of free rRNA and ribosomes equilibrated against each of the four buffers are given in Fig. 2. In the ethidium concentration range employed, free rRNA in MTK 1-10-100 reached full saturation, binding 0.037 ethidium molecules per nucleotide, i.e., one per 27 nucleotides, or 59 per molecule of 16 S rRNA. In the other buffers free rRNA did not reach full saturation but approached it closely enough to show that the results would be similar, about 50 to 80 sites per molecule of 16 S rRNA. The ribosomes, on the other hand, did not approach saturation, and bound less ethidium than did free rRNA at all ethidium concentrations examined. Two firm observations emerge from these data: (a) free 16 S rRNA has about 50-80 intercalation sites per molecule when equilibrated in these buffers; (b) 30 S ribosomes bind much less ethidium than free rRNA at equal ethidium concentrations.

Further information can be obtained by plotting the data according to Scatchard (34). Fig. 3 shows Scatchard plots of the data for RNA and

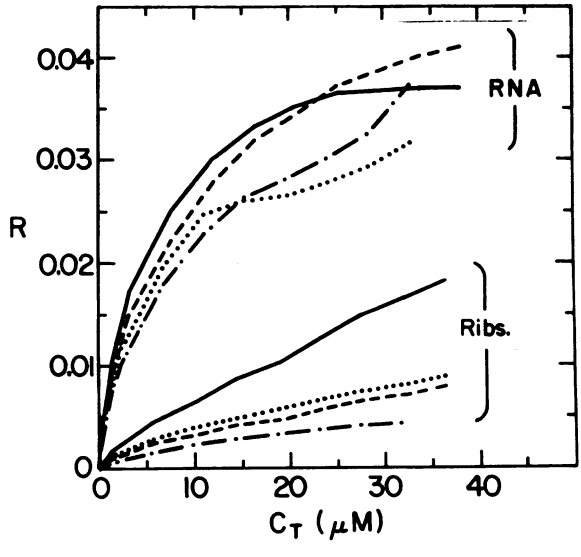

Figure 2.

BINDING OF ETHIDIUM

R: Moles of ethidium bound per mole of nucleotide.

C_T: Total ethidium concentration.

MTK 1-10-20. . . . .
MTK 1-10-100_____
MTK 7-10-300_ _ _ _
MTK 20-10-300__.__.

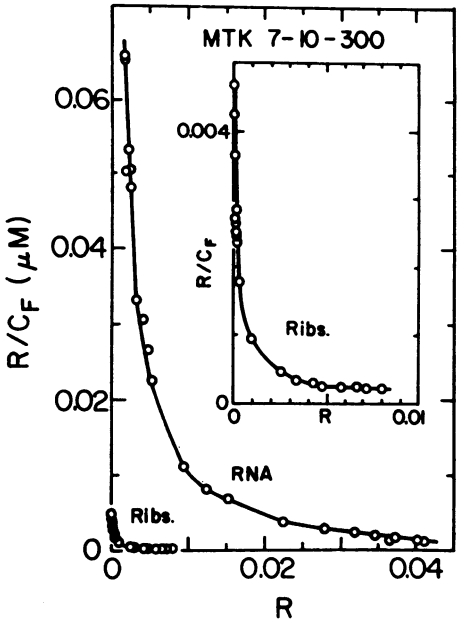

Figure 3.

SCATCHARD PLOTS OF ETHIDIUM BINDING IN MTK 7-10-300

R: Moles of ethidium bound per mole of nucleotide.

C_F: Concentration of unbound ethidium.

Inset: Ribosome curve plotted to an expanded scale.

ribosomes equilibrated against MTK 7-10-300. The other buffers gave similar plots. As is the case with large nucleic acids and nucleoproteins, the plots are bent (e.g., 6, 35-40). In the present case, the points at the upper and lower extremities of the plots fell on well-defined straight lines (Fig.3).

Values for a binding constant, K, and number of sites, N, can be obtained from the slope and R intercept, respectively, of a linear Scatchard plot. For a system with a single type of non-interacting binding site, the plot is a single straight line that gives true values of K and N. This is not the case for more complex multiple-site systems such as nucleic acids and nucleoproteins. In such cases, however, if the different K values are far enough apart, values can be extracted from straight-line portions of the plot that are related to binding constants and site concentrations (41). Without insisting on the absolute validity of these numbers, it is possible to use them to compare different systems (see e.g., ref. 15).

Such data are shown in Table 4. K_1 and N_1 are taken from the upper ends of Scatchard plots and appear to be related to a small number of high-affinity sites. K_2 and N_2 are taken from the lower ends of the plots and would be related to a much larger number of lower-affinity sites of unknown homogeneity. When the data for free rRNA and ribosomes are compared, a

Table 4. ETHIDIUM BROMIDE INTERCALATION

MTK Buffer	Association constants (μM^{-1})		Number of binding sites per ribosome (per 1600 nucleotides)	
	K_1	K_2	N_1	N_2
		16S rRNA		
1-10-20	32	0.24	2.6	53
1-10-100	83	0.25	4.3	62
7-10-300	21	0.13	7.5	72
20-10-300	63	0.14	2.1	62
		30S Ribosomes		
1-10-20	18	0.021	0.40	34
1-10-100	14	0.012	0.61	101
7-10-300	24	0.018	0.35	32
20-10-300	12	0.040	0.30	13

consistent pattern is seen. All four parameters (K_1, N_1, K_2, N_2) are reduced in the ribosome, but the major changes occur in the number of strong binding sites (N_1) and in the binding constants of the weaker sites (K_2). These data indicate that free rRNA contains a small number of strong binding sites that for the most part have been abolished or blocked in the ribosome. The rest of the binding sites in free rRNA (which comprise nearly the total number of sites) are characterized by a lower average binding constant. A large fraction of these weaker sites are present and accessible in the ribosome, but their binding constants are further reduced by a factor of nearly ten.

Since ribosomal RNA appears to have the same amount of base-paired and stacked structure whether free in solution or in the ribosome, it would have the same number of potential ethidium-binding sites in both states. However, this binding potential is depressed in the ribosome with respect to both the number of sites and, especially, their binding constants. This must be due to the presence of the proteins and their effect, reinforced by bound Mg^{++}, in folding the RNA into the more compact configuration demanded by the structure of the ribosome. It is known that ribosomal proteins displace ethidium from rRNA (42). In addition, the greater crowding together of different parts of the rRNA chain may reduce access to binding sites. Further, it is likely that the geometry of ethidium-binding regions is altered, when the rRNA is packed into the ribosome, to a geometry that is

less perfectly adapted to binding the ligand. Also, when a molecule of ethidium is intercalated into a stack of bases or base-pairs, the stack becomes longer and twists (8); RNA in the ribosome is known to have a more stable conformation than free rRNA and would resist this deformation more strongly. Differences in bound Mg^{++} in the four equilibration buffers did not seem to influence ethidium binding significantly; at least, it is difficult to find a meaningful correlation in the data of Table 4. The effect of bound Mg^{++} in this instance appears to be a general one operating in all the buffers, where it reinforces the effect of the proteins in packing the rRNA into its more compact form in the ribosome.

A number of techniques used to study macromolecular conformation have not shown either quantitative or qualitative conformational differences between the compact ribosomal form of rRNA and its free form. The use of ethidium as a probe for this purpose has provided convincing evidence for configurational differences between the two forms.

CONCLUSION

Although the ribosomal RNA is folded into a more compact form when it interacts with the ribosomal proteins and is incorporated into the ribosome, it is generally accepted that the amount of secondary structure in the RNA does not change; and certain techniques sensitive to macromolecular conformation show no change. Our observations show that the two probes, Mg^{++} ions and ethidium bromide, interact with rRNA differently when the RNA is in the ribosome than when it is free, and thereby indicate that details of the structure of the RNA are changed when it is incorporated into the ribosome.

ACKNOWLEDGEMENTS

We are grateful to Dr. Arie Nissenbaum for his instruction and help in the atomic absorption magnesium assays.

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