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*RENATURATION OF TRANSFER RIBONUCLEIC ACIDS THROUGH SITE BINDING OF MAGNESIUM**

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The structural integrity and biological activity of many proteins are known to depend on their content of one or a very few tightly bound low-molecular-weight moieties, often metal ions. There is a growing appreciation of a strong analogy between the factors that determine and affect the molecular structure of proteins and the corresponding factors for nucleic acids. Thus, despite wide differences in the chemical nature of the monomers of which they are formed, the major determinant of their native conformation is their primary structure.[1-3] The bonding forces and environmental factors on which depend the integrity of their conformations also appear to be the same, and they are responsive to similar denaturants.[4] The analogy is particularly striking in the case of sRNA,[5] whose small size is conducive to a unique macromolecular architecture containing elements of both secondary and tertiary structure[6, 7] (rather than to the constellation of statistical structures likely for ribosomal, viral, and messenger RNA's). The finding reported here, that several sRNA's (when prepared by conventional methods involving denaturing steps) require the incorporation of site-bound Mg^{++} or some other divalent cation in order to be able to express their amino acid acceptor activities, makes the analogy seem even more plausible.

While the involvement of Mg^{++} in the structure and function of sRNA has been

suspected, until now there has been no direct indication that Mg^{++} serves any other purpose than to neutralize charge repulsions between the anionic sites (phosphate groups) in the polynucleotide backbone. Thus, it has been reported that Mg^{++} binding to polynucleotides of up to one equivalent of Mg^{++} per phosphate can occur and that the pH dependence of Mg^{++} binding by sRNA and denatured DNA are similar.[8] Moreover, from studies of the activity coefficients of Na^{+} and Mg^{++} salts of native and denatured DNA in the absence of excess salt, it has been concluded that the modes of binding of Na^{+} and Mg^{++} by both forms of DNA are quite similar, although Mg^{++} is more firmly bound.[9]

Indeed, it is this great affinity for Mg^{++} by polynucleotides[10] that has been generally assumed as the explanation for the marked increase in T_m and sharpening of the thermal denaturation profile of sRNA brought about by low concentrations of this ion.[11–14] It has also been reasoned that this tightness of binding of Mg^{++} to the phosphate groups strengthens the secondary structure of the macromolecule, thereby causing the marked diminution in sensitivity of sRNA to endonuclease degradation.[14, 15] A similar explanation has been offered for the Mg^{++} inhibition of the reaction of formaldehyde with sRNA.[16]

Current information on the participation of Mg^{++} in the function of sRNA is indecisive. The formation of aminoacyl-sRNA involves two distinct steps. While the first of these, the formation of an enzyme-aminoacyl-adenylate complex, is known to be Mg^{++}-dependent, the second step, involving transfer of the aminoacyl group from the complex to sRNA, was first reported to show no Mg^{++} requirement.[17–19] It has more recently been reported that there *is* an absolute requirement for divalent cations in aminoacyl transfer to sRNA by a rat liver threonyl-sRNA synthetase if the sRNA preparations employed have been previously dialyzed against EDTA.[20]

The denaturation of sRNA has been judged to be fully reversible when assessed by a variety of physical-chemical and biological criteria.[21] Moreover, in a recent study[3] a comparison was made between the physical properties and amino acid accepting activities of sRNA prepared by means that never lead to its denaturation (designated *native* sRNA) with sRNA prepared by conventional procedures that usually include denaturing steps (designated *conventional* sRNA). No significant differences were observed. Such observations have been taken as evidence that the molecular structure of biologically active sRNA is determined by the interplay of the environmental conditions of temperature and ionic strength with the intramolecular interactions dictated only by the nucleotide sequence of the macromolecule. Notwithstanding these observations that sRNA is apparently readily and completely renaturable at moderate ionic strengths and temperatures, the present work will show that a biologically inactive form of sRNA may be stabilized under such conditions (designated *denatured* sRNA), and that biological activity may be restored through the introduction of one or a very few site-bound Mg^{++} or other divalent cations (designated *renatured* sRNA).

The point of departure of the present work was an attempt to make a more detailed comparison than was done previously[3] of the amino acid accepting activities of native *yeast* sRNA with conventional *yeast* sRNA. Whereas in the earlier work no difference had been observed between the amino acid accepting activities of these two preparations for five amino acids (alanine, glycine, phenylalanine, tyro-

sine, valine), a comparison made for several additional amino acids revealed that the native preparation contained a substantially higher accepting activity for leucine. In the course of seeking to understand the basis for this difference, it was discovered that the activity of native sRNA could be brought down to the level of conventional sRNA by exposing it to chelating agents at elevated temperatures. In turn, it was found that conventional sRNA could have its level of activity for leucine acceptance raised to the level present in native sRNA by exposing it to Mg^{++} at elevated temperatures. It was further possible to show that the continuing presence of the introduced divalent cations is necessary for the biological activity of this and some other individual sRNA species in *yeast* and *Escherichia coli*.

Methods.—sRNA preparations: Native *yeast* sRNA was made as previously described.[3] A conventional yeast sRNA preparation was made according to Holley[22] and further purified by means of repeated phenol extraction, ammonium sulfate fractionation (43–95%), and exhaustive dialysis against EDTA and distilled water.[23] *E. coli* sRNA, a product of Schwarz BioResearch, Inc., was dialyzed against 0.01 *M* Tris-HCl + 0.001 *M* Na_2-EDTA, pH 8.0. sRNA concentrations were determined spectrophotometrically using an extinction coefficient of $A_{258}^{1\%} = 215$.[24] A molecular weight of 26,500 was assumed for both *yeast* and *E. coli* sRNA.[24, 25]

Assay procedure: Amino acid acceptor activity was assayed by a modification of the method of Loftfield and Eigner.[26] The assay mixture contained 10 μM Tris-HCl, pH 7.5; 1 μM $MgCl_2$; 1 μM K-ATP; 2 μM KCl; 0.05 μM K_2-EDTA; 0.01 μM amino acid (0.05-0.5 μc C^{14} or 0.5–2 μc H^3); 0.15 mg enzyme; and a limiting amount of sRNA in a total volume of 0.1 ml. The mixture was incubated at 25°, for 20 min unless otherwise stated. The reaction was stopped by the addition of 20 vol 5% cold TCA, and the precipitates collected on membrane filters, washed with 100 vol 5% TCA, dried, and counted at known efficiency in a Packard Tri-Carb scintillation counter. The data are given as mole amino acid accepted per mole unfractionated sRNA.

Preparations of activating enzymes from *yeast* or *E. coli* were obtained by disintegrating frozen cells (grinding under liquid nitrogen), extracting with 1.2 vol of 0.06 *M* KCl + 0.01 *M* Mg-acetate + 0.01 *M* Tris-HCl, pH 7.8 + 0.006 *M* mercaptoethanol, and then centrifuging first at 15,000 × *g* for 20 min, followed by 105,000 × *g* for 90 min. The supernatant was passed through a Sephadex G-25 column equilibrated with 0.05 *M* Tris-HCl, pH 7.5 and the synthetases recovered as the nonretarded fraction.

Fractionation of yeast sRNA: Countercurrent distribution was performed in a 340-tube (10/10 ml) Craig-Post type machine at 24°. Transfers (430) were made in a phosphate buffer-formamide-isopropanol two-phase solvent system,[27] to which 0.001 *M* EDTA had been added. The EDTA caused no significant change in the distribution of sRNA between the phases. Fractions were recovered according to Apgar *et al.*,[28] dissolved in 0.01 *M* Tris-HCl + 0.001 *M* EDTA, pH 8.0, and heated at 60° for 5 min.

Results.—Effect of Mg^{++} on leucine acceptor activity of yeast sRNA: When conventional yeast sRNA is pretreated by heating at 60° for 5 min in the presence and absence of Mg^{++}, the subsequent kinetics of leucine loading at 25° shown in Figure 1 are observed. It is evident that the saturation level of leucine acceptor activity is substantially higher when the sample is pretreated with Mg^{++}. This shows that conventional sRNA contains a fraction of leucyl-sRNA which can be renatured to a biologically active form by pretreatment with Mg^{++} at 60°. However, during the bioassay at 25°, where Mg^{++} is present, no renaturation occurs, as indicated by the plateau between 15 and 50 min.

Figure 2 shows the dependence of the leucine acceptor activity of conventional *yeast* sRNA when pretreated in the presence of Mg^{++} at different temperatures and then assayed at 25°. It is shown that up to 30° such pretreatment has no effect, but that from 30 to 50° Mg^{++} serves to raise the level of leucine acceptor activity. One control in this experiment was to pretreat conventional sRNA at different tem-

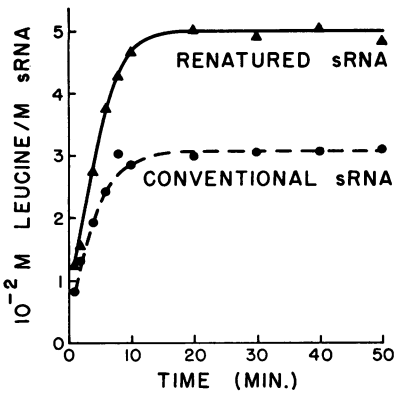

FIG. 1.—Kinetics of loading of *yeast* sRNA with C^{14}-leucine. sRNA's were incubated at 25° under standard assay conditions. At different times aliquots were removed and precipitated with TCA. ▲—▲, sRNA pretreated with 0.01 *M* $MgCl_2$ + 0.01 *M* Tris HCl, pH 8.0 + 0.001 *M* Na_2-EDTA at 60° for 5 min (renatured); ●----●, same pretreatment, but in the absence of Mg^{++} (conventional).

peratures in the absence of Mg^{++}. This had no effect on the level of leucine acceptor activity. A second control involved the pretreatment of renatured sRNA in the presence of Mg^{++} at different temperatures before assaying for leucine acceptor activity. In this case the already renatured sRNA has the same activity as that of native sRNA, and as has been observed as well with native sRNA, there is no additional effect of further exposure to Mg^{++} at the different temperatures. The drop in the level of leucine acceptor activity above 70° may be ascribed to degradation reactions known to be catalyzed at high temperatures by divalent cations.[29]

Effect of Mg^{++} on renaturation of individual yeast leucyl-sRNA's: In order to relate the enhancement of leucine acceptor activity achieved by Mg^{++} pretreatment to discrete sRNA species, a countercurrent fractionation of conventional *yeast* sRNA was performed, and individual fractions were assayed for acceptor activity before and after pretreatment with Mg^{++} at 60°. Figure 3 shows that before pretreatment two leucyl-sRNA peaks (I and II) are in evidence and that a third discrete leucyl-sRNA component (III) becomes apparent after the pretreatment. (By contrast, no difference was observed in the distribution of valyl-sRNA before and after similar pretreatment.) The renatured leucyl-sRNA is obviously an independent component, unrelated to the other two, since its appearance is not accompanied by a diminution in the original two present in conventional sRNA. In fact, the new leucyl-sRNA fraction quantitatively accounts for the difference in levels of loading activity between native or renatured and conventional sRNA's. It is clear, then, that the difference between leucine acceptor activity in conventional and native or renatured sRNA is this particular leucyl-sRNA component. The inactivity in conventional sRNA of this renaturable leucyl-sRNA is apparently not due to its being in some aggregated state, since prior to the pretreatment the peak

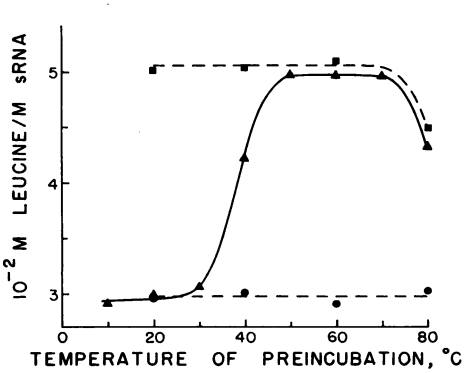

FIG. 2.—Effect of pretreatment with Mg^{++} at different temperatures on leucine acceptor activity of conventional *yeast* sRNA. ▲—▲, Aliquots of an sRNA solution (1 mg/ml) were heated with 0.02 *M* $MgCl_2$ + 0.01 *M* Tris-HCl, pH 8.0 + 0.001 *M* Na_2-EDTA for 5 min at different temperatures, cooled, and assayed under standard conditions; ●----●, same pretreatment, but in the absence of Mg^{++}. ■----■, sRNA was heated to 60° for 5 min with Mg^{++} as above and cooled. Aliquots were subjected to further heating at different temperatures.

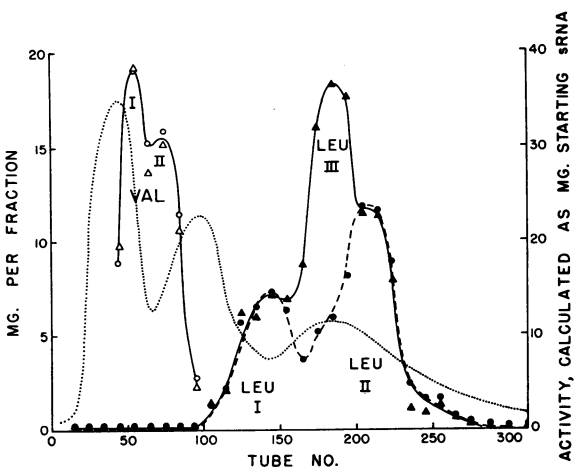

FIG. 3.—430-Transfer countercurrent distribution of 500 mg conventional yeast sRNA; ....., mg sRNA; ●----●, leucine acceptor activity; ▲—▲, leucine acceptor activity after pretreatment of sRNA with Mg^{++} at 60°; O—O, valine acceptor activity; and △—△, valine acceptor activity after pretreatment of sRNA with Mg^{++} at 60°.

fraction in which it occurs exhibits an $S^{\circ}_{20,w}$ of 3.9, compared to a mean value of 4.0 for the unfractionated sRNA from which it was obtained. Moreover, there was no indication of faster sedimenting components in the sedimenting boundary.

Comparison of native and renatured sRNA: In connection with Figure 2 it was stated that the saturation level of leucine acceptor activity in native and renatured sRNA's are the same. It has further been observed that the pretreatment of native sRNA in the absence of Mg^{++} and presence of 0.001 *M* EDTA at 60° leads to a level of leucine acceptor activity equal to that of conventional *yeast* sRNA. While these findings would suggest the identity of renatured sRNA with native sRNA, the following observations indicate that this is not so. Native sRNA dialyzed for a period of 24 hr against 0.001 *M* EDTA at 4° is unaffected. By contrast, renatured sRNA exposed to the same concentration of EDTA at 4° loses all renaturable activity within 1 hr. Thus, these two forms, while possessing equal acceptor activity, are distinguishable. In this connection, renaturation with Co^{++}, Ca^{++}, Mn^{++}, or various diamines in place of Mg^{++} was partially achieved.

Evidence for renaturation through Mg^{++} site binding: The pretreatment with Mg^{++} could induce renaturation merely by facilitating the folding of sRNA into some unique conformation or, instead, by being directly incorporated into the renatured sRNA molecule. Evidence for the latter hypothesis has been provided by the following results. When citrate (a weaker chelating agent, capable of removing all Mg^{++} bound to phosphate groups in native and denatured DNA[9]) was added in excess to renatured sRNA at 0°, a slow loss of leucine acceptor activity (over several hours) occurred down to the level of conventional sRNA. In a second experiment renatured leucyl-sRNA (countercurrent fraction Leu III) was cooled to 0°, excess citrate added, and the solution immediately passed through a Sephadex G-50 column (equilibrated with 0.05 *M* KCl + 0.01 *M* Tris-HCl, pH 7.5) at 4° to remove all Mg^{++} citrate and excess citrate. The resulting sRNA showed after 3 hr at 0° essentially the same activity as the starting renatured material. However,

an aliquot, to which citrate was added to 0.001 *M*, gradually lost activity over the same period. As the citrate did not significantly change the ionic strength and pH of the sRNA solution, its effect is ascribable to its chelating property. Consequently, the loss of activity of renatured sRNA on addition of citrate indicates the presence of site-bound Mg^{++} in the renatured sRNA.

Other renaturable sRNA's in yeast and E. coli: To screen for the presence of other sRNA's renaturable by pretreatment with Mg^{++} at 60°, conventional *yeast* and *E. coli* sRNA's were pretreated at that temperature in the presence and absence of 10^{-2} *M* Mg^{++}, then cooled to 25° and assayed for amino acid acceptor activities. The results obtained to date are summarized in Table 1. Of the amino acids tested in *yeast*, renaturable sRNA's have been detected for leucine and arginine. For the latter, preliminary analyses of individual countercurrent fractions indicate a picture comparable to that observed for leucyl-sRNA's; that is, pretreatment with Mg^{++} reveals an arginine-accepting peak not otherwise apparent (in addition to two conventional arginyl-sRNA peaks[30]). In *E. coli* renaturable sRNA's have been found for glutamine, glutamic acid, histidine, tryptophan, and possibly leucine.

Discussion.—The discovery that a site-bound divalent cation(s) is an integral component of the biologically active form of several sRNA's adds a new dimension to considerations of the structure and biological role of this class of macromolecules.[31] It is too early to decide whether this phenomenon is restricted to a select class of sRNA's, or instead represents a general occurrence, of which the examples uncovered represent extreme cases that have been detected due to the difficulty with which the cation is reinserted. If the latter is the case, as seems likely, it would mean that

TABLE 1

AMINO ACID ACCEPTOR ACTIVITIES OF YEAST AND *E. coli* sRNA'S WHEN ASSAYED WITH HOMOLOGOUS ENZYMES

	M Amino Acid Accepted/*M* sRNA			
	Yeast sRNA		*E. coli* sRNA	
Amino acid	Conventional	Renatured	Conventional	Renatured
Alanine	0.024	0.023	0.036	0.036
Arginine	*0.029*	*0.041*	0.043	0.043
Aspartic acid	0.019	0.019	0.019	0.019
Asparagine	0.024	0.021	0.003	0.003
Cysteine	0.003	0.003	0.007	0.008
Glutamic acid	0.003	0.003	*0.002**	*0.005**
Glutamine	—	—	*0.011*	*0.026*
Glycine	0.062	0.062	0.046	0.046
Histidine	0.018	0.018	*0.021*	*0.029*
Isoleucine	0.020	0.018	0.019	0.019
Leucine	*0.029*	*0.050*	*0.071*	*0.080*
Lysine	0.027	0.027	0.035	0.035
Methionine	—	—	0.039	0.039
Phenylalanine	0.012	0.012	0.022	0.022
Proline	—	—	0.029	0.029
Serine	0.035	0.034	0.023	0.023
Threonine	0.007	0.007	0.022	0.022
Tryptophan†	0.027	0.029	*0.022*	*0.030*
Tyrosine	0.025	0.025	0.018	0.018
Valine	0.019	0.019	0.024	0.024

Renaturation was attempted by heating sRNA (1 mg/ml) for 5 min at 60° in the presence of 0.02 *M* $MgCl_2$ + 0.01 *M* Tris-HCl + 0.001 *M* Na_2-EDTA, pH 8.0.

* Increasing the concentration of glutamic acid tenfold in the assay mixture produced a threefold increase in apparent acceptor activity. However, the relative difference between the two sRNA samples did not change appreciably.

† For tryptophanyl-sRNA, cellulose membrane filters were replaced with glass filters in order to reduce background due to absorption of free tryptophan on the membrane (Gartland and Sueoka, personal communication).

most sRNA's in the absence of the special cation have a conformation at moderate conditions of temperature and ionic strength that is already favorable to the binding of the cation, but that sRNA's requiring renaturation at 60°, in the absence of the cation assume a structure at lower temperatures that precludes binding. A higher temperature is therefore needed to disrupt this unfavorable conformation, so that binding can occur, thereby enabling the development of the unique conformation requisite for biological activity.

The very fact that it has been possible to trap an sRNA in a biologically inactive form demonstrates, for the first time, that a unique structure is an absolute requirement for biological activity. This finding also adds weight to our previous arguments that sRNA is endowed with unique *tertiary* structure.[2, 6, 7]

If site-bound divalent cations are requisite components of all biologically active forms of sRNA, an explanation may be provided for the puzzling difference in the way low Mg^{++} concentration affects sRNA on the one hand, and ribosomal and viral RNA's on the other. In the case of the latter, 10^{-3} M Mg^{++} raises T_m to that observed with 1 M Na^+,[10, 32] but the sigmoidal character of the absorbance-temperature profile is retained. By contrast, whereas 1 M NaCl has the same effects on the profile of sRNA,[25] Mg^{++} increases T_m, but also causes the profile to take on a cooperative character like that of DNA. This change may occur because site-bound Mg^{++} stabilizes helical regions above the temperature of stability characteristic of the individual regions in the absence of the cation. Moreover, the conformation in the presence and absence of the site-bound cation may be different in some sRNA's.

Summary.—A *yeast* leucyl transfer ribonucleic acid can be stabilized in two different forms. One is biologically inactive, as measured by its ability to accept leucine, but can be converted into the active form by exposure to Mg^{++} at elevated temperatures; the activity is lost again on exposure to chelating agents. It is shown that one (or a few) site-bound divalent cation(s) is an obligatory part of the biologically active structure for this molecule. Evidence is given for the occurrence of several other transfer ribonucleic acids of this type in *yeast* and *Escherichia coli*.

We gratefully acknowledge the expert technical assistance of Mrs. Mary Geroch. We are indebted to Schwartz BioResearch, Inc., for the *E. coli* sRNA, and to P. Spadafora of Standard Brands, Inc., for the log phase yeast from which native sRNA was prepared.

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[5] The following abbreviations have been used: sRNA = transfer ribonucleic acid; DNA = deoxyribonucleic acid; RNA = ribonucleic acid; TCA = trichloroacetic acid; ATP = adenosine triphosphate; EDTA = ethylenediaminetetraacetate; A_{258} = absorbance at 258 mμ; $S^0_{20,w}$ = sedimentation coefficient at infinite dilution corrected to 20° in water; DEAE = diethylaminoethyl; T_m = temperature at midpoint of a thermal denaturation profile.

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*TWO INTERCONVERTIBLE FORMS OF TRYPTOPHANYL SRNA IN E. COLI**

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Involvement of sRNA as the adaptor between an RNA template and a polypeptide chain[1, 2] predicts that this class of molecules should have at least three functional sites. One site confers specificity for recognition by the correct aminoacyl-sRNA synthetase, another serves as an anticodon for hydrogen bonding to specific