Acquisition of Native Conformation of Ribosomal 5S Ribonucleic Acid from *Escherichia coli*. Hydrodynamic and Spectroscopic Studies on the Unfolding and Refolding of Ribonucleic Acid†

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ABSTRACT: In a continuing effort to decipher the molecular mechanism of ribosome self-assembly [e.g., Dunn, J. M., & Wong, K.-P. (1979) *Biochemistry 18*, 4380–4385], the mechanism of folding of 5S RNA was investigated by unfolding and refolding studies using several physical techniques including circular dichroism (CD), UV absorption spectroscopy, and sedimentation velocity analysis to monitor various conformational changes. The 5S RNA was unfolded by using 6 M urea and EDTA, and an unfolded state was characterized in which the base pairing was found to be disrupted, but extensive base stacking remained. The unfolded 5S RNA was then refolded upon removal of urea and EDTA by dialysis against a reconstitution buffer both with and without Mg^{2+}, and the refolded states were characterized. The results indicate that under the proper conditions, 5S RNA refolds to a conformation and overall shape very similar to the native conformation. These results indicate that the nucleotide sequence in 5S RNA contains the necessary information to direct the folding of the RNA into its native conformation. The presence of an appropriate concentration of Mg^{2+} and an incubation at 60 °C are required for the correct refolding, since omission of either one results in a renatured 5S RNA whose conformation is quite different from the native one.

Ribosomes are complex cellular ribonucleoprotein particles containing many different proteins and a few ribosomal RNAs arranged in a rather specific manner to function as a precise machinery for protein synthesis. The successful in vitro reconstitution of functional ribosomes solely from their biomolecular component proteins and RNAs (e.g., Nomura, 1973; Nierhaus, 1979) suggested that the information required for the self-assembly process may lie on the unique conformations of the RNAs and the proteins under the conditions of reconstitution. These unique conformations of proteins and RNAs may provide the bases for specific molecular recognitions and interactions.

Previous studies in this laboratory have shown that many ribosomal proteins free in solution possess unique conformation and conformational stability which are sensitive to change of environmental conditions (Allen & Wong, 1978a,b). This is exemplified by a careful study of the shape and conformation of ribosomal protein L7/L12 (Wong & Paradies, 1974; Luer & Wong, 1979) and its conformational stability (Luer & Wong, 1980). These studies also indicate that the formation of the unique conformation of protein L7/L12 is directed solely by the amino acid sequence since the unfolding of the protein by various denaturants is completely reversible. The conformation of ribosomal proteins also changes extensively during the course of in vitro ribosomal reconstitution as shown by difference CD[1] spectropolarimetry and chemical iodination (Dunn & Wong, 1979b; Fox & Wong, 1978). Such conformational changes seem to generate new ribosomal protein binding sites on the reconstitution intermediate particles for ribosome assembly to proceed further (Dunn & Wong, 1979a,b).

The most significant finding is the demonstration by a number of solution physical studies that ribosomal 16S RNA free in solution has a rather compact shape and unique conformation which is very similar to the conformation of the 16S RNA bound with proteins in the 30S ribosome (Allen & Wong, 1978c, 1979a,b). This conclusion is contrary to an earlier proposal that the ribosomal RNA free in solution exists in rather extended conformation and that binding of proteins organized the extended RNA molecule into a compact structure (Kurland, 1974). Instead, it demonstrates, for the first time, that the free 16S RNA in solution under the conditions of reconstitution already assumes a three-dimensional structure which is very similar to the RNA structure in the ribosome and contains at least some protein binding sites. This conclusion has been established independently by other investigators using other methods (Vasiliev et al., 1978) and confirmed by more recent hydrodynamic measurements (Tam et al., 1981). It has also been shown that this unique conformation of the 16S RNA undergoes subtle localized conformational rearrangements during the course of assembly with concomitant stability changes (Dunn & Wong, 1979a,c). Whether this unique compact preassembly conformation is formed from the nascent polynucleotide chain and directed by the nucleotide sequence alone or through other more complicated mechanisms is not known.

Many possibilities exist as to the manner in which 5S RNA or other ribosomal RNAs fold into their native conformation. One might initially argue that 5S RNA does not contain a unique three-dimensional structure and that it functions in the assembly of the 50S subunit simply through interactions with specific nucleotides which make up the sequence of 5S RNA. However, the fact that denatured forms of 5S RNA have been characterized which cannot be incorporated into partially reconstituted 50S subunits argues against this possibility (Reynier et al., 1967; Aubert et al., 1973). Various structural studies [for review, see Erdmann (1976)] including the more recent work (e.g., Österberg et al., 1976; Fox & Wong, 1979a)

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[1] Abbreviations: CD, circular dichroism; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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have suggested the existence of a highly organized conformation of 5S RNA. Precursor molecules of 5S RNA have been reported (Ginsburg & Steitz, 1975; Jordan et al., 1971; Feunteun et al., 1972; Griffin & Bailie, 1973), and the additional bases at each end of the 5S RNA molecule which are found in these precursor molecules could be involved in the inducement and/or stabilization of the native structure. In this case, the native structure of 5S RNA would not form without these additional bases. Another possibility as to the manner in which 5S RNA acquires its native conformation is the ribonucleotide sequence in 5S RNA could itself give rise to the native structure without the extra sequences found in the precursor molecules. Still another possibility is the interaction of specific proteins with 5S RNA resulting in conformational changes that give rise to the native structure. And, of course, any combination of the above could constitute the actual mechanism in which 5S RNA acquires its native three-dimensional structure. In the case of many proteins, the formation of the native unique compact conformation is directed solely by the amino acid sequence of the polypeptide chain (e.g., Anfinsen & Scheraga, 1975; Wong & Tanford, 1973).

The present paper reports results on in vitro unfolding and refolding studies of ribosomal 5S RNA in an attempt to understand how the native (preassembly) conformation of ribosomal RNA is formed. We have chosen the 5S RNA for this investigation because of its relative simplicity and the considerable amount of information available on this RNA [for a review, see Erdmann (1976)], as well as the availability of a proposed three-dimensional model based on extensive physical and chemical data (Fox & Wong, 1979a). The results show that unfolded ribosomal 5S RNA can be refolded to a native-like conformation and indicate that the ribonucleotide sequence of this small RNA directs the folding of the RNA chain to its native conformation. The results also indicate that the presence of Mg^{2+} and an incubation at elevated temperature are required for the 5S RNA to refold to its native conformation.

Materials and Methods

Buffer. TMK_{360} buffer contained 0.01 M Tris, 0.02 M $MgCl_2$, and 0.36 M KCl, pH 7.6. This is the reconstitution buffer, and it represents functionally significant ionic conditions which have been used in the successful reconstitution of biologically active *B. stearothermophilus* 50S ribosomal subunits (Nomura & Erdmann, 1970) and the reconstitution of specific 5S RNA–protein complexes from *E. coli* and *B. stearothermophilus* (Horne & Erdmann, 1972). TK_{360} buffer contained 0.01 M Tris and 0.36 M KCl, pH 7.6.

Purification of E. coli 5S RNA. Ribosomal 5S RNA was purified by gel filtration on Sephadex G-100 according to the procedure of Erdmann and co-workers (Erdmann et al., 1971) using the modifications described previously (Fox & Wong, 1979a).

Hydrodynamic and Spectroscopic Methods. Ultraviolet absorption spectroscopy and circular dichroism measurements were obtained as described previously (Fox & Wong, 1979a).

Sedimentation velocity experiments were performed with a Beckman Model E analytical ultracentrifuge employing UV absorption optics. Exposed ultraviolet-sensitive film was scanned with an ISCO Model 1310 gel scanner at a wavelength of 546 nm. 5S RNA in a 7 M urea buffered solution (7 M urea, 0.01 M EDTA, and 0.25 M Tris, pH 8.9) was analyzed in a 12-mm double sector cell at 60 000 rpm. 5S RNA solutions which had been treated with 7 M urea, followed by dialysis into TMK_{360} and TK_{360} buffers, and untreated solutions of 5S RNA were analyzed in 30-mm single sector cells run at 48 000 rpm at 25 °C. The observed sedimentation coefficients were corrected for the density and viscosity of water at 20 °C by using the measured density and viscosity of the respective buffered solutions (Owens et al., 1980).

Polyacrylamide Gel Electrophoresis. The purity of the 5S RNA preparation was ascertained by using polyacrylamide gel electrophoresis according to Richards & Lecanidou (1971). The gels used contained 10% of a mixture of acrylamide and *N,N'*-methylenebis(acrylamide) (Bis) in the ratio 19:1. A Tris buffer (pH 7.8) was used which was 0.05 M Cl^- (adjusted by adding concentrated HCl acid) and 0.02 M in *N,N,N'N'*-tetramethylethylenediamine (TEMED). Approximately 2 μg of 5S RNA in 20 μL of running buffer containing 5% sucrose was applied to the gel (5 × 50 mm) and electrophoresis was carried out for 60 min at 5 mA/gel. Gels were stained in a solution of 1% Pyronine Y and 15% acetic acid and destained in 15% acetic acid. Gels were scanned at 475 nm by using a Gilford 240 spectrophotometer equipped with a Model 2410 linear transporter.

In order,to determine whether or not our preparations of purified 5S RNA consisted of intact 5S RNA, we carried out electrophoresis under denaturing conditions to disrupt the base pairing, thus enabling a determination of the number of single-stranded polyribonucleotides present. If our 5S RNA preparation had a nick in the polyribonucleotide chain, then the electrophoretic pattern should consist of two bands and, if more than one nick, then several bands. (Our analysis, of course, could not detect the presence of a nick in our preparations of 5S RNA which would give rise to two polyribonucleotides of equal length.) Electrophoresis was carried out as described above with the addition of varying concentrations of urea to the gel solution. The gels contained 15% of a mixture of acrylamide and Bis in the ratio 19:1 and were run at 5 mA/gel for 3 h. Gels were also run at a pH of 3.5 according to Rosen et al. (1975). The electrophoretic buffer used here consisted of 0.025 M citric acid (pH 3.5).

Unfolding and Refolding Studies. To 0.02 mL of a stock solution of 5S RNA (0.14 mg/mL) in 0.25 M Tris (pH 8.9) was added 4 mL of a solution of 7.35 M urea, 0.01 M EDTA, and 0.25 M Tris (pH 8.9). The resulting solution contained 5S RNA at a concentration of 0.007 mg/mL in 7 M urea, 0.01 M EDTA, and 0.25 M Tris (pH 8.9). A solution of 5S RNA in 0.25 M Tris (pH 8.9) was also prepared in an identical manner as the control, i.e., the addition of 4 mL of 0.25 M Tris (pH 8.9) to 0.2 mL of 5S RNA stock solution. The refolded 5S RNA was obtained by removing urea and EDTA through exhaustive dialysis of the unfolded RNA against a 1000-fold excess of reconstitution buffers without Mg^{2+} (TK_{360}) or with Mg^{2+} (TMK_{360}). Complete removal of urea and EDTA was ascertained by refractive index measurement of the solutions. By use of a Bausch & Lomb refractometer sensitive to 0.0001 refractive index unit, this technique can detect a concentration of urea as low as 0.02 M. After exhaustive dialysis, the refractive index readings of the refolded 5S RNA solutions were the same as those of the respective TK_{360} and TMK_{360} buffers. The renatured solutions of 5S RNA were then examined in order to determine if the 5S RNA had refolded to its starting structure. This was followed by incubating at 60 °C for 10 min and obtaining additional sedimentation velocity and spectroscopic data. The spectroscopic data were obtained by incubating the 5S RNA solutions in the cell block without removing the solution from the cell and without disturbing the position of the cell in the cell block.

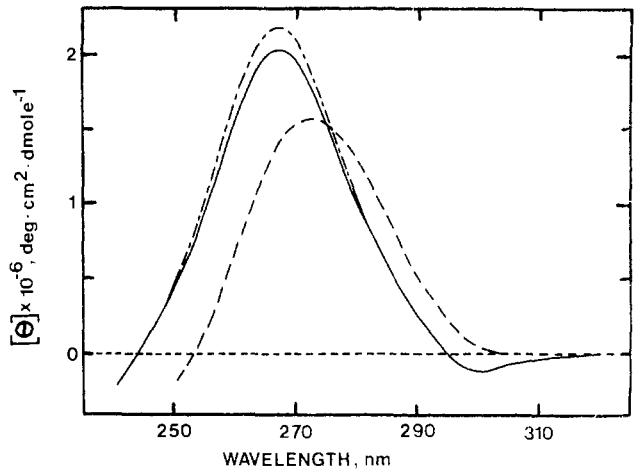

FIGURE 1: Near-UV CD spectra of 5S RNA in EDTA. Symbols: solid curve, 5S RNA in TMK_{360} buffer, pH 7.6; dashed curve, 5S RNA in 0.01 M Tris and 0.4 mM EDTA at 50 °C; broken curve, 5S RNA refolded in TMK_{360} after EDTA and 50 °C treatment. All spectra were obtained at room temperature.

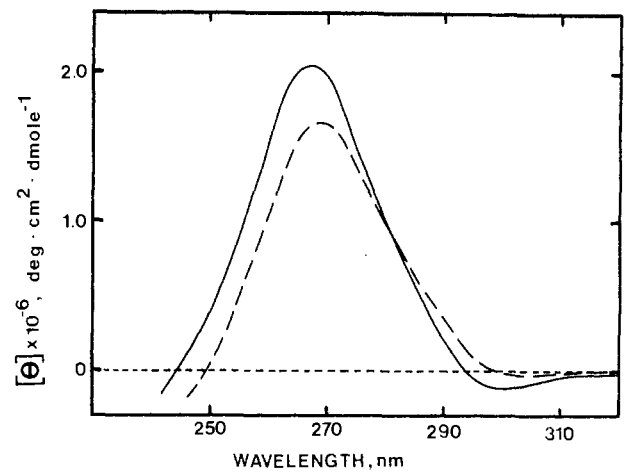

FIGURE 2: Near-UV CD spectra of 5S RNA in urea and EDTA. Symbols: solid curve, 5S RNA in TMK_{360}, pH 7.6; dashed curve, 5S RNA in 7 M urea, 0.01 M EDTA, and 0.25 M Tris, pH 8.9. All spectra were obtained at room temperature.

The effect of EDTA alone on the CD spectrum of 5S RNA was studied by adding 0.4 mM EDTA to 0.00092 mM 5S RNA in 0.01 M Tris (pH 8.0). This sample was then dialyzed against TMK_{360} buffer, and CD spectra were obtained before and after an incubation at 60 °C.

Miscellaneous. The concentration of 5S RNA was determined by using $A_{260nm}^{1mg/mL}$ equal to 21.9 (Österberg et al., 1976). All pH measurements were obtained at room temperature with a Radiometer Model PHM 64 research pH meter using a combined glass electrode. All water used was deionized and double distilled. All glassware was acid washed, and centrifuge tubes and other vessels that could not be acid washed were soaked in a solution containing 2% $NaDodSO_4$ and 2% bentonite to avoid RNase degradation.

Results

Electrophoretic Analyses. Purified 5S RNA was run in 15% polyacrylamide gels under denaturing conditions. One gel consists of 15% polyacrylamide and 4 M urea at pH 7.8 and another gel consists of 15% polyacrylamide and 6 M urea at pH 3.5. In 4 M urea at neutral pH, one broad major band can be seen along with many smaller bands which appear to occur in pairs. In 6 M urea at pH 3.5, the same preparation and same stock solution of purified 5S RNA migrate as one major band with only slight traces of two other bands migrating ahead of the major band. The disappearance of the many bands seen in the gel run at neutral pH in 4 M urea as a result of running the same sample under increased denaturing conditions (low pH and 6 M urea) strongly suggests that these multiple bands arise from partially denatured forms of 5S RNA. The single band observed when electrophoresis is carried out at 6 M urea and pH 3.5 indicates that our preparation of 5S RNA consists of a single polyribonucleotide. If our preparation of 5S RNA contained a nick or several nicks, more than one band would be present in the gels run under these denaturing conditions. Thus, our results on the refolding of 5S RNA can be considered as the refolding of a single polyribonucleotide chain and not as a more complicated process involving the refolding of several polyribonucleotides.

Unfolding–Refolding Studies Using EDTA. 5S RNA was denatured upon the addition of 0.4 mM EDTA in 0.01 M Tris (pH 8.0). A slight decrease in the magnitude and a red shift of 2 nm in the 268-nm CD peak were observed at 37 °C (data not shown). Increasing the temperature to 50 °C resulted in a 23% decrease in this peak with a red shift of 4 nm as shown by the dashed curve in Figure 1. It should be noted that in TMK_{360} buffer 5S RNA at 50 °C exhibited only an 8% decrease in the magnitude of the 268-nm CD peak with no red shift (unpublished results). The sample was then dialyzed overnight against TMK_{360} buffer, and another CD spectrum was obtained as shown by the broken curve in Figure 1. When the sample was heated at 60 °C for 15 min, the CD curve showed a decrease in the 268-nm CD peak, resulting in a curve which was indistinguishable from the CD curve for the native 5S RNA in TMK_{360} buffer.

Unfolding Studies Using EDTA and Urea. A more complete study of the unfolding and refolding of 5S RNA was performed by unfolding the 5S RNA with both EDTA and urea. 5S RNA was unfolded with 7 M urea and 0.01 M EDTA, and the resulting denatured state was characterized spectroscopically. The denaturants were then removed by exhaustive dialysis against TK_{360} and TMK_{360} buffers. Dialysis into these two buffers provides a means of determining the role of Mg^{2+} in the refolding process. The refolded samples were then incubated at 60 °C for 10 min in order to determine the influence of temperature on the refolding process.

The effect of 7 M urea and 0.01 M EDTA on the conformation of 5S RNA was studied in order to determine the degree of unfolding of 5S RNA as a result of such treatment. Figure 2 shows the change in the near-UV CD spectrum of 5S RNA upon adding 7 M urea and 0.01 M EDTA. A decrease in molar ellipticity from 2.08×10^6 to 1.68×10^6 (19%) was observed in the 268-nm CD peak along with a red shift of approximately 1 nm. The magnitude of the 300-nm trough changed drastically, decreasing from a molar ellipticity of 0.12×10^6 to 0.03×10^6. The trough and the crossover points were red shifted by approximately 5 nm.

The effect of the 7 M urea and 0.01 M EDTA treatment on the UV absorption spectrum of 5S RNA was studied. A hyperchromic effect of 15.4% of the original absorption peak at 260 nm was observed along with a slight red shift.

Figure 3 shows the effect on the UV absorption melting profile of 5S RNA. The melting profile in TMK_{360} buffer exhibits a sigmoidal-shaped cooperative curve typical of double-stranded helical nucleic acids, while the melting profile after the 7 M urea and 0.01 M EDTA treatment exhibits a linear noncooperative increase in absorbance upon increasing temperature.

The sedimentation velocity analysis of 5S RNA in 7 M urea, 0.01 M EDTA, and 0.25 M Tris (pH 8.9) is shown in Table

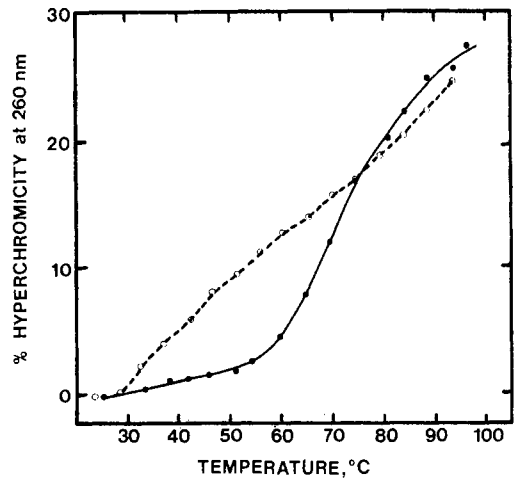

FIGURE 3: UV absorption melting profile of unfolded 5S RNA. Symbols: solid curve, 5S RNA in TMK_{360}, pH 7.6; dashed curve, 5S RNA in 7 M urea, 0.01 M EDTA, and 0.25 M Tris, pH 8.9.

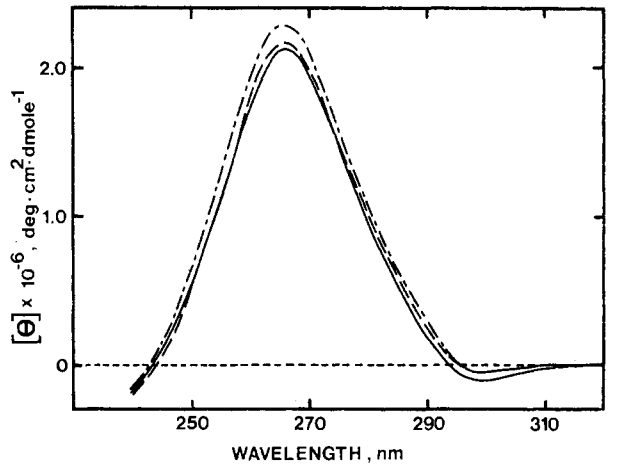

FIGURE 4: Near-UV CD spectra of 5S RNA upon unfolding and refolding in TK_{360}. Symbols: solid curve, 5S RNA in TK_{360}, pH 7.6; broken curve, 5S RNA which has been unfolded and refolded in TK_{360}, pH 7.6; dashed curve, 5S RNA which has been refolded and after an incubation at 60 °C for 10 min. All spectra were obtained at room temperature.

Table I: Sedimentation Coefficient ($s_{20,w}$) and Frictional Coefficient Ratio (f/f_{min}) of Native, Unfolded, and Refolded 5S RNA

5S RNA samples	$s_{20,w}$ (S)	f/f_{min}
native, with 20 mM Mg^{2+} (TMK_{360} buffer)	5.3	1.4
native, no Mg^{2+} (TK_{360} buffer)	4.7	1.6 (1.64)
unfolded, 7 M urea, 0.01 M EDTA, 0.25 M Tris (pH 8.9)	4.3	1.8
refolded in the presence of Mg^{2+} (TMK_{360} buffer)	5.3	1.4
refolded in the presence of Mg^{2+} (TMK_{360} buffer) and incubated at 60 °C for 10 min	5.3	1.4
refolded, no Mg^{2+} (TK_{360} buffer)	4.9	1.6 (1.58)
refolded, no Mg^{2+} (TK_{360} buffer) and incubated at 60 °C for 10 min	3.8	2.0

I. Under these conditions, 5S RNA sediments with an $s_{20,w}$ value of 4.3 S, giving a frictional ratio (f/f_{min}) of 1.8. In comparison to native 5S RNA in TMK_{360} buffer, the 7 M urea and 0.01 M EDTA denatured 5S RNA shows a decrease in sedimentation coefficient and an increase in frictional coefficient ratio, indicating an unfolded and more extended structure.

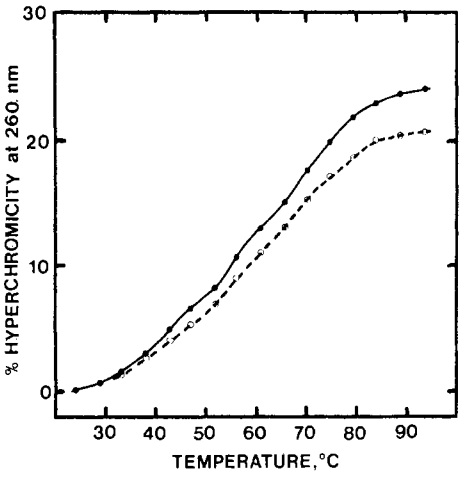

FIGURE 5: UV absorption melting profiles of 5S RNA upon unfolding and refolding in TK_{360} buffer. Symbols: solid curve, unfolded 5S RNA in TK_{360}, pH 7.6; dashed curve, 5S RNA which has been unfolded and refolded in TK_{360}, pH 7.6.

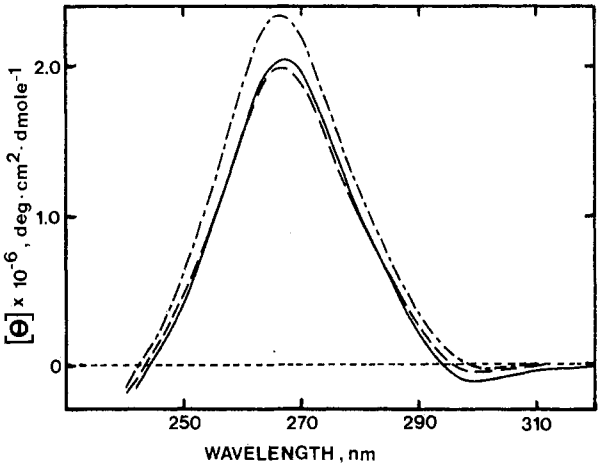

FIGURE 6: Near-UV CD spectra of 5S RNA upon unfolding and refolding in TMK_{360}. Symbols: solid curve, untreated 5S RNA in TMK_{360}, pH 7.6; broken curve, 5S RNA which has been unfolded and refolded in TMK_{360}, pH 7.6; dashed curve, 5S RNA which has been refolded after an incubation at 60 °C for 10 min. All spectra were obtained at room temperature.

Refolding and the Role of Mg^{2+} and Temperature. Figure 4 shows the near-UV CD spectrum of the denatured 5S RNA after exhaustive dialysis into the Mg^{2+}-free TK_{360} buffer. The broken curve is the spectrum of the refolded 5S RNA at room temperature, and the dashed curve is the spectrum after incubation at 60 °C for 10 min. The solid curve is the CD spectrum of native 5S RNA in TK_{360} buffer. After dialysis into TK_{360} buffer, the peak maximum at 267 nm is 7% larger than that of native 5S RNA. The 300-nm trough is about half the value of that of the native 5S RNA and slightly red shifted. Upon incubation at 60 °C for 10 min, the peak maximum decreases to an ellipticity only slightly larger (1%) than that of native 5S RNA, and the 300-nm trough remains unchanged.

In Figure 5, the UV absorption melting profile of the refolded 5S RNA in TK_{360} buffer is compared with that of native 5S RNA in TK_{360} buffer. The native 5S RNA exhibits an ~24% hyperchromicity, whereas the refolded 5S RNA in TK_{360} buffer exhibits an ~21% hyperchromicity.

The sedimentation velocity results of denatured 5S RNA after dialysis into TK_{360} buffer are included in Table I. The refolded 5S RNA sediments with an $s_{20,w}$ value of 4.9 S, which is slightly larger than the 4.7S value observed for untreated 5S RNA in TK_{360} buffer. In both cases, these sedimentation

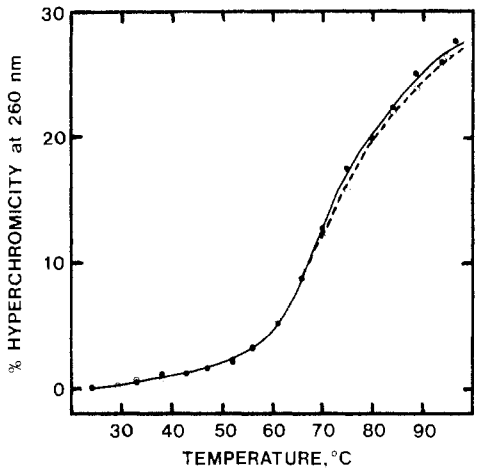

FIGURE 7: UV absorption melting profiles of 5S RNA upon unfolding and refolding in TMK_{360}. Symbols: solid curve, native 5S RNA in TMK_{360}, pH 7.6; dashed curve, 5S RNA which has been unfolded and refolded in TMK_{360}, pH 7.6.

coefficients give rise to an f/f_{min} value of 1.6. After incubation at 60 °C for 10 min, the $s_{20,w}$ decreases to a value of 3.8 S with an f/f_{min} of 2.0.

Figure 6 shows the CD spectra on the refolding of the denatured 5S RNA upon exhaustive dialysis into the Mg^{2+}-containing TMK_{360} buffer. Before incubation at 60 °C, the CD spectrum shows a 14% increase in the 268-nm peak concurrent with a blue shift of 1 nm. After incubation at 60 °C for 10 min, the peak maximum decreases to a value 3% smaller than the native peak maximum, and the 300-nm trough increases to approximately half the value of the native 5S RNA.

The UV absorption melting profile for the unfolded 5S RNA in TMK_{360} buffer is compared with that of native 5S RNA in Figure 7. The melting profiles are almost identical, indicating that 5S RNA has refolded to essentially the same amount of ordered structure as present in native 5S RNA.

The sedimentation velocity results indicate that, upon refolding into TMK_{360} buffer, 5S RNA sediments with an $s_{20,w}$ value of 5.3 S. This value is the same as that of native 5S RNA in TMK_{360} buffer (see Table I). After an incubation at 60 °C for 10 min, the sedimentation coefficient remains unchanged.

Discussion

Urea and EDTA Appear To Unfold the 5S RNA to a Single-Stranded RNA Which Contains Very Few Base-Paired Double-Helical Structures but Is Extensively Stacked. The conformational change in 5S RNA as a result of the removal of Mg^{2+} with EDTA is similar to the change observed in ribosomes upon the same treatment (Wong & Dunn, 1974). However, the change in the CD spectrum is less drastic in the case of 5S RNA until the temperature is increased to well above 37 °C. The change observed in the CD spectrum indicates a destruction of base pairing and base stacking in 5S RNA (e.g., Brahms & Mommaerts, 1964; Vournakis & Scheraga, 1966; Yang & Sameijima, 1969). The CD spectrum obtained after dialysis into TMK_{360} buffer is similar to that of the native sample, but the magnitude of the 268-nm CD peak is 7% larger. Upon incubation at 60 °C for 10 min, the CD spectrum becomes indistinguishable from that of the native 5S RNA, indicating a return to the native conformation. Thus, the unfolding effects of EDTA and heat seem to be reversible upon dialysis into TMK_{360} buffer followed by an incubation at 60 °C for 10 min. Treatment of 5S RNA with 7 M urea and 0.01 M EDTA results in a decrease and red shift in the 268-nm CD peak, indicating a disruption of base pairing and base stacking. The large decrease in the 300-nm trough indicates a disruption of the tilting of the bases in base-paired regions (Bloomfield et al., 1974).

The linear noncooperative melting profile indicates an absence of base pairing, as the curve completely lacks cooperativity (Bloomfield et al., 1974). This melting profile also indicates that considerable stacking interactions are still present in the unfolded state, as a large linear hyperchromic effect is observed upon increasing temperature. Such an increase indicates an unstacking of single-stranded RNA (Stevens & Rosenfeld, 1966; Blum et al., 1972). The disruption of base pairing is also indicated by the hyperchromicity change in the UV absorption spectrum upon urea and EDTA treatment. Boedtker & Kelling (1967) showed that the reaction of 5S RNA with formaldehyde to completely disrupt hydrogen bonding indicated that the hyperchromicity due to base pairing was 18.5%. This agrees favorably with the 15.4% hyperchromicity reported here.

The decrease in the corrected sedimentation coefficient from 5.3 S in TMK_{360} buffer to 4.3 S in 7 M urea, 0.01 M EDTA, and 0.25 M Tris (pH 8.9) indicates an unfolding of the molecule to a more extended molecule and is consistent with the spectroscopic data, indicating a disruption of base pairing.

The unfolded state of 5S RNA can thus be defined as a structure in which the base pairing has been disrupted but extensive base stacking remains. This conclusion is consistent with the spectroscopic data, as mentioned above, and also consistent with the denaturing properties of EDTA and urea. Wong & Dunn (1974) showed that the addition of EDTA to ribosomes at 37 °C produced drastic structural changes in the base pairing and base stacking of the RNA. The addition of urea has been shown to result in the denaturation of nucleic acids [Rice & Doty, 1957; Geiduschek & Holtzer, 1958; Levine et al., 1963].

Presence of Mg^{2+} Is Critical to the Correct Refolding of 5S RNA. Removal of the urea and EDTA by dialysis into TK_{360} buffer, followed by an incubation at 60 °C for 10 min, results in a conformation similar to that of the native state, as determined by CD spectroscopy. The similarity in the two CD spectra indicates the geometry of the bases is nearly the same. However, the melting profile for the refolded sample exhibits a decrease in hyperchromicity with respect to the native 5S RNA, indicating a decrease in the amount of base pairing and/or base stacking. This difference might be due to the presence of tightly bound Mg^{2+} in the native sample which is still bound to the molecule even in TK_{360} buffer. This tightly bound Mg^{2+} would not be present in refolded 5S RNA since the molecule has previously been unfolded in the presence of EDTA which would remove all Mg^{2+}. We have also shown (J. W. Fox and K.-P. Wong, unpublished results) the effect of Mg^{2+} in stabilizing and inducing base stacking and/or base pairing in 5S RNA, and its absence in the refolded sample could explain the decrease in the observed hyperchromicity.

The corrected sedimentation coefficient for the refolded 5S RNA is somewhat larger than that of the native RNA in TK_{360} buffer, but the frictional ratios are the same. This difference may also be due to the presence of tightly bound Mg^{2+} in native 5S RNA and its absence in the refolded sample. The decrease in the $s_{20,w}$ value to 3.8 S and increase in the frictional ratio to 2.0 after incubating at 60 °C for 10 min indicate the formation of an unfolded and more extended conformation as compared with the native state. This drastic change in the sedimentation coefficient and frictional ratio is not observed in the presence of Mg^{2+}.

High Temperature Is Also Needed for the Correct Refolding of the 5S RNA in the Presence of Mg^{2+}. The CD spectrum of 5S RNA refolded into TMK_{360} buffer after an incubation at 60 °C for 10 min indicates a conformation similar to native 5S RNA in TMK_{360} buffer. This same effect was also seen in the case of 5S RNA which had been unfolded with only EDTA and refolded into TMK_{360} buffer. After 5S RNA is incubated at 60 °C for 10 min, the CD curve becomes indistinguishable from that of the native 5S RNA (see Figure 2). Richards et al. (1973) reported a similar effect of incubating denatured 5S RNA at 60 °C in the presence of Mg^{2+} which resulted in a conversion to a renatured form as judged by polyacrylamide gel electrophoresis.

The melting profile of the refolded 5S RNA, as shown in Figure 7, is almost identical with the native sample, indicating a similarity in the nature and extent of ordered structure. The melting technique, of course, effectively measures the hyperchromicity of the refolded form after the 60 °C incubation.

The sedimentation velocity results indicate that the refolding of 5S RNA into TMK_{360} buffer results in an overall shape which is the same as untreated 5S RNA. However, contrary to the spectroscopic results, this occurs without an incubation at 60 °C. After the incubation, the sedimentation coefficient remains unchanged.

These observations lead to several conclusions concerning the unfolding and refolding of 5S RNA. In the absence of Mg^{2+}, 5S RNA refolds to a structure quite different from the native 5S RNA. In the presence of Mg^{2+}, 5S RNA refolds to a structure which has the same hydrodynamic shape as that of native 5S RNA, and after an incubation at 60 °C for 10 min, the spectroscopic parameters indicate a return to a conformation which is very similar to that of native 5S RNA.

Significance to RNA Folding and Ribosome Assembly. The results obtained here which show that 5S RNA appears to unfold into a conformation similar to the newly synthesized 5S RNA and may refold to its native conformation led us to conclude that *the polynucleotide sequence in 5S RNA contains the necessary information to direct the folding of the RNA molecule into its unique secondary and tertiary structure under the proper conditions.* It should be added that the refolding process may not be totally complete, but the small differences between the refolded state and the native 5S RNA are very close to the limits of detection of the spectroscopic measurements.

The refolding of 5S RNA from the 7 M urea and 0.01 M EDTA state is similar to the manner in which newly synthesized polyribonucleotides fold into their native conformation. The growing polyribonucleotide extends away from the RNA polymerase–DNA complex (Chamberlin, 1976). As the polyribonucleotide grows, it gains the potential for the formation of base pairing and other interactions which make up the native conformation. Stacking interactions are present in the 7 M urea and 0.01 M EDTA unfolded 5S RNA and are probably present in the growing polyribonucleotide, as the hydrophobic bases will tend to interact with each other instead of the hydrophilic environment. Also, the presence of stacking interactions in single-stranded polyribonucleotides is supported by a considerable amount of spectroscopic data (Bloomfield et al., 1974). The refolding of the unfolded 5S RNA into the correct base pairing and overall conformation, therefore, represents a good approximation to the folding of the newly synthesized polyribonucleotide.

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References

Allen, S. H., & Wong, K.-P. (1978a) *Biochemistry 17*, 3971–3978.

Allen, S. H., & Wong, K.-P. (1978b) *Biophys. J. 23*, 359–373.

Allen, S. H., & Wong, K.-P. (1978c) *J. Biol. Chem. 253*, 8759–8766.

Allen, S. H., & Wong, K.-P. (1979a) *J. Biol. Chem. 254*, 1775–1777.

Allen, S. H., & Wong, K.-P. (1979b) *Arch. Biochem. Biophys. 195*, 112–120.

Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205–300.

Aubert, M., Bellemare, G., & Monier, R. (1973) *Biochimie 55*, 135–142.

Bloomfield, V. A., Crothers, D. M., & Tinoco, I., Jr. (1974) in *Physical Chemistry of Nucleic Acids*, Harper & Row, New York.

Blum, A. D., Uhlenbeck, O. C., & Tinoco, I., Jr. (1972) *Biochemistry 11*, 3248–3256.

Boedtker, H., & Kelling, D. G. (1967) *Biochem. Biophys. Res. Commun. 29*, 758–766.

Brahms, J., & Mommaerts, W. F. H. M. (1964) *J. Mol. Biol. 10*, 73–88.

Chamberlin, M. J. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 17–67, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Dunn, J. M., & Wong, K.-P. (1979a) *J. Biol. Chem. 254*, 7705–7711.

Dunn, J. M., & Wong, K.-P. (1979b) *J. Biol. Chem. 254*, 7712–7716.

Dunn, J. M., & Wong, K.-P. (1979c) *Biochemistry 18*, 4380–4385.

Erdmann, V. A. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 18*, 45–90.

Erdmann, V. A., Doverer, H. G., & Sprinzl, M. (1971) *Mol. Gen. Genet. 114*, 89–94.

Feunteun, J., Jordan, B. R., & Monier, R. (1972) *J. Mol. Biol. 70*, 465–474.

Fox, J. W., & Wong, K.-P. (1978) *J. Biol. Chem. 253*, 18–20.

Fox, J. W., & Wong, K.-P. (1979a) *J. Biol. Chem. 254*, 10139–10144.

Fox, J. W., & Wong, K.-P. (1979b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 228.

Geiduschek, E. P., & Holtzer, E. P. (1958) *Adv. Biol. Med. Phys. 6*, 431–551.

Ginsburg, D., & Steitz, J. A. (1975) *J. Biol. Chem. 250*, 5647–5654.

Griffin, B. E., & Bailie, D. L. (1973) *FEBS Lett. 34*, 273–279.

Horne, J. R., & Erdmann, V. A. (1972) *Mol. Gen. Genet. 119*, 337–344.

Jordan, B. R., Forget, B. G., & Monier, R. (1971) *J. Mol. Biol. 55*, 407–421.

Kurland, C. G. (1974) *J. Supramol. Struct. 2*, 178–188.

Levine, L., Bordon, J. A., & Jencks, W. P. (1963) *Biochemistry 2*, 168–175.

Luer, C. A., & Wong, K.-P. (1979) *Biochemistry 18*, 2019–2027.

Luer, C. A., & Wong, K.-P. (1980) *Biochemistry 19*, 176–183.

Nierhaus, K. H. (1979) in *Ribosome: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davis, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 267–294, University Park Press, Baltimore, MD.

Nomura, M. (1973) *Science (Washington, D.C.) 179*, 864–873.

Nomura, M., & Erdmann, V. A. (1970) *Nature (London) 228*, 744-747.
Österberg, R., Sjöberg, B., & Garrett, B. A. (1976) *Eur. J. Biochem. 68*, 481-487.
Owens, D. P., Fox, J. W., & Wong, K.-P. (1980) *Biochem. Biophys. Res. Commun. 96*, 685-691.
Reynier, M., Aubert, M., & Monier, R. (1967) *Bull. Soc. Chim. Biol. 49*, 1205-1219.
Rice, S. A., & Doty, P. (1957) *J. Am. Chem. Soc. 79*, 3937-3947.
Richards, E. G., & Lecanidou, R. (1971) *Anal. Biochem. 40*, 43-71.
Richards, E. G., Lecanidou, R., & Geroch, M. E. (1973) *Eur. J. Biochem. 34*, 262-267.
Rosen, J. M., Woo, S. L. C., Holder, J. W., Means, A. R., & O'Malley, B. (1975) *Biochemistry 14*, 69-78.
Stevens, C. L., & Rosenfeld, A. (1966) *Biochemistry 5*, 2714-2721.
Tam, M. F., Dodd, J. A., & Hill, W. E. (1981) *J. Biol. Chem. 256*, 6430-6434.
Vasiliev, V. D., Selinvanova, O. M., & Koteliansky, V. E. (1978) *FEBS Lett. 95*, 273-276.
Vournakis, J. N., & Scheraga, H. A. (1966) *Biochemistry 5*, 2997-3006.
Wong, K.-P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8518-8523.
Wong, K.-P., & Dunn, J. M. (1974) *FEBS Lett. 44*, 50-54.
Wong, K.-P., & Paradies, H. H. (1974) *Biochem. Biophys. Res. Commun. 61*, 178-184.
Yang, J. T., & Sameijima, T. (1969) *Prog. Nucleic Acid Res. Mol. Biol. 9*, 223-300.

Mechanistic Studies of Ribonucleic Acid Renaturation by a Helix-Destabilizing Protein†

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ABSTRACT: The ability of a nucleic acid helix-destabilizing protein from calf thymus, UP1, to facilitate renaturation of yeast $tRNA_3^{Leu}$ and *Escherichia coli* 5S RNA is shown to be a consequence of the protein's ability to bind stoichiometrically to single-stranded polynucleotide regions. A comparison of the inhibitory effect of different homopolymers on UP1-induced renaturation of $tRNA_3^{Leu}$ does not indicate significant base specificity in UP1 binding, and a 3′-5′ ribose phosphate polymer devoid of heterocyclic bases inhibits as well as the homopolynucleotides. These inhibition studies also show that UP1 requires polynucleotide segments of at least three phosphate residues to bind. Mg^{2+} (which is required for the stabilization of native $tRNA_3^{Leu}$) dissociates complexes of UP1 with inactive tRNA, and since the RNAs in those complexes lack a substantial amount of secondary structure, it can upon dissociation readily refold into the native structure. A semiquantitative treatment of UP1–RNA interaction is developed that suggests that only a small number (approximately six) of protein molecules are bound to $tRNA_3^{Leu}$ in the complex while analysis of the inhibition studies suggests that these UP1 molecules are not bound in a highly cooperative manner.

A number of nucleic acid helix-destabilizing proteins (Karpel et al., 1974, 1975a,b, 1976) (formerly termed unwinding proteins) can accelerate the renaturation of metastable, biologically inactive tRNA (Lindahl et al., 1966; Fresco et al., 1966; Adams et al., 1967; Webb & Fresco, 1973; Ishida & Sueoka, 1967) and 5S RNA conformers (Aubert et al., 1968; Richards et al., 1973). The spontaneous renaturations of yeast $tRNA_3^{Leu}$ and 5S RNA are processes with activation energies of about 60 (T. Lindahl, G. Payne, R. L. Karpel, and J. R. Fresco, unpublished results) and 65 kcal/mol (Richards et al., 1973), respectively. These large barriers suggest that renaturation likely involves the disruption of many "incorrect" base pairs in the inactive conformer to allow correct refolding to the native biologically functional conformer (Fresco et al., 1966; Adams et al., 1967; Webb & Fresco, 1973; Uhlenbeck et al., 1974; Wong et al., 1973). It seems reasonable, therefore, that the accelerating action of nucleic acid helix-destabilizing proteins in these cases is related to their ability to promote disruption of base-paired regions by tightly but transiently binding to segments of the RNA that are single stranded or become so upon interaction with the protein.

Although there is at present no evidence that inactive tRNAs occur in vivo, the process by which helix-destabilizing proteins accelerate RNA renaturation is viewed as a model of the mechanism whereby several different RNA conformational changes occur in the cell (Karpel et al., 1974). For example, the complementary pairing between *Escherichia coli* mRNA and 16S rRNA during protein synthesis (Shine & Delgarno, 1974) may be dependent on the helix-destabilizing activities of proteins S1 and IF3 (VanDieijen et al., 1976). The mechanism of denaturation and formation of *intra*molecular tRNA structure in the presence of a helix-destabilizing protein may thus serve as a model for the analogous melting and formation of *inter*molecular base pairing during initiation of protein synthesis as well as for other processes involving RNA conformational interchange (Karpel, 1981).

This study examines the mechanism by which the calf thymus protein UP1 (Herrick & Alberts, 1976a) brings about the renaturation of RNA, using that of $tRNA_3^{Leu}$ as a model. It explores the binding stoichiometry between protein and RNA, with a view toward understanding the nature of the

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