Evidence that 16S RNA from E.coli can assume two different biologically active conformations

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ABSTRACT

We have recently shown¹ that 16S RNA can be extracted from 30S ribosomes by an acetic acid-urea precipitation procedure which yields RNA capable of binding 13 individual ribosomal proteins. This is in contrast to phenol extracted 16S RNA which can specifically associate with only 7 proteins²⁻⁷. In the experiments reported here, we demonstrate that the difference in protein binding capacities is due to a relatively more "open" configuration possessed by the acetic acid-urea 16S RNA.

Under identical conditions, acetic acid-urea 16S RNA is more susceptible to limited T₁-RNase digestion than is phenol-16S RNA. In addition, acetic acid-urea RNA shows a relatively slower electrophoretic mobility. The observable difference in conformation between the two types of RNA is lost by storage at -70°C. This loss is accompanied by a reduction in protein binding capacity of the acetic acid-urea 16S RNA.

INTRODUCTION

Several independent laboratories have established that E. coli 16S RNA specifically binds seven 30S ribosomal proteins ^{2,3,4,5,6,7}. However, all of these investigations were conducted with 16S RNA prepared by the traditional technique of phenol extraction⁸. We have found a new method of 16S RNA purification¹, utilizing acetic acid precipitation in the presence of 1 M urea. This new procedure yields RNA capable of individually and specifically binding a total of 13 ribosomal proteins. The most direct interpretation of this discovery is that the acetic acid-urea 16S RNA maintains a substantially different 3-dimensional structure than that possessed by phenol extracted 16S RNA.

In this communication we report experiments which attempt to determine any differences in conformation between acetic acid-urea 16S RNA and phenol 16S RNA. We have found several important structural parameters which are significantly

different for the two types of RNA. On the basis of these observations, we propose that acetic acid-urea 16S RNA exhibits a considerably more "open" or extended tertiary structure in comparison to phenol 16S RNA which possesses a relatively more "closed configuration."

MATERIALS AND METHODS

Buffers

- I 0.03 M Tricine (Sigma Chem.Co.), 0.02 M Magnesium Acetate, pH 8.0.
- II 0.03 M Tricine, 0.4 M Potassium Chloride, 0.02 M Mg Acetate, 0.001 M Dithiothreitol (Nutritional Biochem. Corp.), pH 7.4.
- III 0.05 M Tris, 0.2 M Ammonium Chloride, 0.02 M Mg Acetate, 0.002 M Dithiothreitol, pH 7.0.
- IV. 0.15 M NaCl, 15 mM Sodium Citrate, 10 mM Ethylenediamine Tetracetate, pH 7.0.
- V. 0.09 M Tricine, 1.2 M Potassium Chloride, 0.06 M Mg Acetate, 0.001 M Dithiothreitol, pH 7.4.

Preparation of Ribosomes and Ribosomal Proteins

Ribosomes were isolated from E. coli MRE 600 and 30S particles were purified as previously described⁹. The purity of the 30S subunits was routinely checked on 5-20% sucrose gradients and on 10% polyacrylamide gels (protein composition). They were found to contain less than one percent of contaminations with 50S subunits. Ribosomal proteins were extracted from the 30S subunits with 67% acetic acid and fractionated by phosphocellulose (Mannex-P1) chromatography following the procedure described by Hardy et al.¹⁰. The nomenclature of the 30S ribosomal proteins is taken from Wittmann et al.¹¹.

Preparation of Radioactive RNA

E. coli cells were grown in a low phosphate containing medium and labeled with 10 mCi of ³²P according to the procedure of Sanger et al.¹². Crude 70S ribosomes were isolated and the ribosomal RNA was extracted by phenol⁸. The 16S and 23S RNA were fractionated by ultracentrifugation on a 5-20% sucrose gradient.

Isolation of Ribosomal RNA from 30S Ribosomes

The phenol and Na dodecyl sulfate extraction procedure described by Traub et al.⁸ was used without modification to

purify 16S RNA from isolated 30S subunits.

The acetic acid-urea procedure published by Hochkeppel, et al.¹ was used as originally delineated. The RNA is precipitated from 30S ribosomes by a mixture of acetic acid, urea and $MgCl_2$ (final concentrations respectively are 75%, 1.0M and 0.2M). The RNA precipitate is resuspended in buffer II and re-extracted. The precipitate is then washed with a Magnesium-Tricine solution to remove excess acetic acid and urea. Finally the precipitated RNA is dissolved in 0.03 M Tricine, pH 8.0. The quantity of protein which remains associated with the RNA was estimated to be less than 1% for acetic acid-urea 16S RNA as well as for phenol 16S RNA. This estimate was obtained by extracting ribosomes containing radioactive protein (prepared by in vivo labeling). Neither polyacrylamide gels nor composite agarose-polyacrylamide gels of acetic acid-urea 16S RNA and phenol 16S RNA showed any contaminations of 23S RNA.

Formation and Isolation of RNA Protein Complexes

With modifications previously described¹ the reconstitution conditions developed by Traub and Nomura¹³ were used to form specific RNA-protein complexes. The RNA and protein were suspended in buffer II and buffer V respectively. The two suspensions were mixed along with the appropriate amount of water to give a final concentration of salts identical to buffer II. The protein and RNA mixture was incubated at 30°C for 10 minutes and then at 42°C for 30 minutes. The resultant RNA-protein complexes were purified by ultracentrifugation through a layer of 12% sucrose dissolved in buffer II.

Polyacrylamide Gel Electrophoresis of Protein

One dimensional polyacrylamide gel electrophoresis of the proteins extracted from ribonucleoprotein complexes was carried out using the gel system described by Leboy et al.¹⁴ and modified by Voynow and Kurland¹⁵. Samples were prepared for electrophoresis as previously described¹. The amount of protein present in an RNA complex was estimated by scanning the stained gels with a microdensitometer (Gilford Model 2000) at a wavelength of 540 nm. The relative amount of protein stain is expressed as percent of the amount of stain obtained from a known standard protein gel.

Agarose-Polyacrylamide Gel Electrophoresis of RNA

The gel and buffer systems employed for electrophoresis of intact 16S RNA are modifications of those described by Peacock and Dingman¹⁶. The electrode buffer system contained 0.06 M Tricine, 0.06 M Boric Acid and 0.06 M KOH. The final pH was 8.0. The gel mixture was composed of 6 gm sucrose, 1 gm agarose (Sigma Chemical Co.), 6 ml of an acrylamide solution (28.5 gm recrystallized Acrylamide, 1.5 gm Bisacrylamide, 48 gm Urea, dissolved in water to a final volume of 100 ml), made to a volume of 100 ml with water. The resultant concentrations of acrylamide and agarose are 1.8% and 1.0% respectively.

In some experiments two different preparations of RNA were analyzed side by side on the same gel. The gel was partitioned by inserting a small piece of a plastic cover slip into the gel tube prior to the addition of the gel solution.

Sucrose Gradient Ultracentrifugation

Gradients of 5-20% sucrose in buffer II were made for routine sedimentation analysis. Approximately 300 μ g of RNA in a volume of 150 μ l was layered on the gradient and centrifuged for 3-1/2 hours at 55,000 rpm in a SW 56 Beckman rotor. Aliquots of each fraction were measured for radioactivity and absorbance at 260 nm.

Temperature Melting of 16S RNA

Heat denaturation profiles of 16S RNA were determined in buffer II. The absorbance of the RNA was measured in a Gilford spectrophotometer properly insulated. The temperature was increased in 2-3° steps from 25° to 85°C. Each measurement was obtained after a 5 minute equilibration period.

Digestion of 16S RNA with T_1 RNase

Approximately 500 μ g of 16S RNA was digested at 4°C with 5-10 units of T_1 RNase (Sigma Chemical Co.) in buffer II. The hyperchromic shift induced by nuclease attack was measured at 260 nm.

Samples of T_1 RNase digestion products were analyzed by polyacrylamide gel electrophoresis. The RNase reaction was terminated by a warm solution of 1% SDS and 1% Bentonite and then extracted by phenol. The aqueous phase was precipitated by ethanol and the resultant RNA was analyzed by the gel

electrophoresis system of Ehresmann *et al.*¹⁷.

Two-Dimensional Paper Fingerprinting of ^{32}P 16S RNA

The techniques used in the preparation of ^{32}P 16S RNA and the two-dimensional separation of T_1 RNase and alkaline phosphatase digestion products were performed by the methods described by Sanger *et al.*¹⁸.

RESULTS

Sedimentation Behavior of Acetic Acid Urea 16S RNA and Phenol 16S RNA

We have shown¹ that 16S RNA prepared by an acetic acid precipitation technique (hereafter referred to as "acetic acid-urea 16S RNA"), can bind individually a total of 13 ribosomal proteins. However, 16S RNA prepared by the classical phenol extraction procedure ("phenol-16S RNA") can bind only 7 ribosomal proteins⁷. We originally suggested that the difference in protein binding capacity between the two preparations of RNA is due to a variance in the RNA 3-dimensional configuration.

We have examined these two types of RNA preparations by a number of experimental criteria sensitive to alterations in conformation. We analyzed the first parameter by sedimentation velocity. Freshly prepared non-radio-active acetic acid-urea 16S RNA and ^{32}P labeled phenol 16S RNA samples were mixed and layered on a sucrose gradient. After centrifugation the gradients were fractionated and the resultant profiles are shown in Figure 1. Both RNA preparations show essentially identical sedimentation behavior. Thus any difference in the configuration of the RNA molecules derived from the two preparations must be too subtle to be detected by sedimentation velocity analysis. The only difference in the sedimentation profiles of both RNA preparations is an additional small shoulder for acetic acid-urea 16S RNA, which most likely represents aggregated acetic acid-urea 16S RNA molecules. As will be demonstrated later on similar aggregation products can be observed on composite agarose-polyacrylamide gels. Since these bands disappear after preheating the RNA preparation, any kind of 23S RNA contamination is ruled out.

Figure 1 Sedimentation Analysis of the Two Different RNA Preparations. Radioactive (^{32}P , 50,000 c.p.m.) phenol 16S RNA was mixed with non-radioactive acetic acid-urea 16S RNA (300 μg) layered on a 5-20% sucrose gradient in buffer II and centrifuged for two hours at 50,000 r.p.m. The gradient was assayed for radioactivity and absorbance at 260 nm.

Gel Electrophoretic Mobilities of Acetic Acid-Urea and Phenol 16S RNA

Other investigators have found conformational differences in preparations of ribosomal RNA which were detected by agarose polyacrylamide gel electrophoresis but not observed by sedimentation velocity analysis¹⁸. Apparently the electrophoretic mobility of RNA in the gel system developed by Peacock and Dingman¹⁶ is a more sensitive probe of subtle conformational alterations in three dimensional structure. We therefore examined the electrophoretic mobilities of acetic acid-urea and phenol 16S RNA.

Agarose-polyacrylamide gels of the two types of RNA are shown in Figure 2. A gel system was used in which both samples were applied to different sides of the same gel tube ("split gels"). Gel (1) in Figure 2 shows a split gel of the two RNAs. Side (a) is acetic acid-urea 16S RNA and side (b) is phenol

16S RNA. Several bands are seen for acetic acid-urea extracted and one additional for phenol extracted 16S RNA, probably due to the presence of aggregates. The major band, representing over 90% of the RNA applied to the gel, is certainly the monomer form. The major band of acetic acid-urea 16S RNA clearly migrates slower than the major band for phenol 16S RNA. Also shown in Figure 2 is a split gel pattern of the two types of 16S RNA after heating the preparations to 42°C for 10 min. prior to the electrophoresis (gel 2). The heating removes the aggregate forms of the RNA and also shows that the difference we see in RNA conformation by this technique cannot be reversed by temperature¹⁸.

The Sensitivity of Acetic Acid-Urea and Phenol RNA to Limited T₁-RNase

The results of the gel electrophoresis analysis and the sedimentation velocity analysis strongly indicate that, compared to phenol 16S RNA, the RNA prepared by the acetic acid-urea technique has a subtle difference in conformation. The slower mobility exhibited by the acetic acid-urea RNA most likely reflects a more extended structure ("open configuration"). We attempted to determine whether this open conformation of acetic acid-urea 16S RNA would cause it to be relatively more susceptible to limited nuclease digestion.

A large number of different ionic conditions and temperature conditions were examined in an attempt to optimize any difference in susceptibility of the two types of RNA to T₁-RNase digestion. It was found that the high ionic strength buffer used for in vitro reconstitution¹⁴ (buffer II) and the temperature of 4°C revealed the greatest detectable difference in T₁-RNase sensitivity. Figure 4 shows the hyperchromic increase in absorbance at 260 nm that occurs during the nuclease digestion reaction. The acetic acid-urea 16S RNA is considerably more open to the T₁-RNase attack than is the 16S RNA prepared by the phenol method. In contrast, when the two types of RNA are artificially expanded into a more open configuration by exposure to low ionic strength, they show identical sensitivities to T₁-RNase (data not shown). Thus acetic acid-urea 16S RNA has a conformation significantly more open to T₁-RNase digestion than

Figure 2 Agarose-polyacrylamide split gel electrophoresis patterns of (a) acetic acid-urea and (b) phenol 16S RNA. Gel pattern (1) was derived from RNA preparations which were unheated prior to electrophoresis and pattern (2) was obtained from RNA preparations heated to 42°C for 10 minutes in buffer II.

phenol 16S RNA.

Corroboration of this conclusion was obtained from a polyacrylamide gel electrophoresis analysis of the T_1 -RNase digestion products (Figure 4). There are many more T_1 -RNase fragments of high molecular weight present in the products derived from phenol 16S RNA and the products obtained from acetic acid-urea 16S RNA contain several additional small molecular weight bands. Thus the acetic acid-urea 16S RNA must contain several distinct regions which are considerably more open to T_1 -RNase attack than phenol 16S RNA.

The Open Conformation of Acetic Acid-Urea 16S RNA is Unstable

In early experiments we observed that the capacity of acetic acid-urea 16S RNA to bind proteins S3, S5, S9, S11, S12, S13 and S18 is readily lost during storage. On the basis of the above results we presumed that this loss in protein binding ability is a consequence of an alteration in the configuration of the

RNA. We tested this hypothesis by attempting to correlate the loss in protein binding function with a change in conformation as detected by agarose polyacrylamide gel electrophoresis.

Figure 3 Kinetics of T_1 -RNase digestion. The two types of 16S RNA preparations were exposed to 10 units of nuclease at 4°C. The progress of the reaction was followed by measuring the relative increase in absorbance at 260 nm. The amount of RNA used in each experiment was approximately 7.5×10^{-10} Moles. o—o represents acetic acid-urea 16S RNA; o-----o represents phenol 16S RNA.

Aliquots of freshly prepared acetic acid-urea 16S RNA were stored at -70°C in 0.03 M Tricine, pH 8.0. The ability of the RNA to bind a mixture of 30S ribosomal proteins S3, S5, S9, S12, S13 and S18, was studied as a function of storage time. The reconstituted complexes were isolated and examined for protein composition by polyacrylamide gel electrophoresis. The gels were stained with Coomassie Blue and the amount of stain was estimated by a densitometric analysis at wavelength 540 nm. (see Materials and Methods).

The acetic acid-urea 16S RNA showed a time dependent loss in capacity to bind the six proteins studied. Figure 5 gives the percent binding capacity for three of the proteins studied, S3, S5 and S9. The binding sites for all these proteins are lost as a function of storage at -70°C . As a control we

demonstrated that acetic acid-urea 16S RNA is unimpaired in its ability to reassemble with all 21 proteins after a 20 day storage period. The physical ability of the RNA preparation to bind 30S ribosomal proteins was tested and shown on polyacrylamide gels (data not shown). It is interesting that the relative rate of protein binding capacity reduction for the three proteins is different in each case. The protein S3 binding site, for example, is lost much more rapidly than any of the other sites of specific protein interaction. This might indicate that not only is the acetic acid-urea 16S RNA conformation metastable, but that the loss of that conformation is an ordered process.

Figure 4 Polyacrylamide gel electrophoresis patterns of limited T₁-RNase digestion products. Acetic acid-urea RNA products are shown in gel a and phenol-16S RNA digestion products in gel b. Digestion conditions were the same as described in Figure 3, except that the reaction was terminated after a 3 minute incubation by phenol extraction, followed by alcohol precipitation. (see Materials and Methods for details). The amount of RNA used in this experiment was approximately 7.5×10^{-10} moles. For convenience top and bottom of the gel picture were cut off, because they didn't contain any staining material.

The loss of protein binding capacity during storage of acetic acid-urea 16S RNA helps to explain many of the ambiguous results we observed in the early phases of the investigation.

We made numerous attempts to find storage conditions which would prevent this conformational change resulting in the reduction of protein binding sites. We found that storage of the RNA at -70°C in 0.03 M Tricine solution containing 30% ethylene glycol, significantly increases the resistance of the RNA to storage deterioration. For example, after 12 days of storage the binding sites for S12 and S13 lost only 25% of their original binding capacity in ethylene glycol, whereas in our standard storage buffer the loss was 75% of the original activity. A similar effect was observed also for the other protein binding sites studied (S3, S5, S9 and S18). We conclude that 30% ethylene glycol helps to maintain the relatively open structure of acetic acid-urea 16S RNA.

Figure 5 The effect of storage at -70°C on the capacity of acetic acid-urea 16S RNA to bind ribosomal proteins. The progress curves for the loss of binding capacity for proteins S3 (Δ — Δ), S5 (o—o) and S9 (●—●) are presented here as representative of all the proteins tested. Each protein was bound to RNA which had been stored for different periods of time. The complex was purified, the protein extracted and applied to polyacrylamide gels for electrophoresis. The resultant stained protein band was quantitated by densitometry analysis.

The hypothesis which most consistently explained the loss in protein binding function of acetic acid-urea 16S RNA is that the structure it possesses in a fresh preparation can be readily altered during storage. We tested this hypothesis by examining the electrophoretic mobility of the RNA after various periods of storage. Figure 6 shows photographs of split gel electrophoresis patterns of the two types of RNA preparations. The acetic acid-urea 16S RNA undergoes a conformational change during storage as reflected by a relative increase in electrophoretic mobility. After 24 days of storage the RNA has lost all of its capacity to bind proteins S3,S5,S9,S11,S12 and S18 and concomitantly has undergone conformational change such that its electrophoretic mobility is identical to phenol 16S RNA.

Figure 6 Agarose-polyacrylamide split gel electrophoresis patterns of acetic acid-urea 16S RNA (left) and phenol 16S RNA (right) after various periods of storage at -70°C gel (a), 4 days; gel (b), 12 days; gel (c), 16 days; and gel (d), 20 days.

Monovalent Ion Depleted and Activated 30S Ribosomes Contain RNA Molecules with Different Protein Binding Capacities

It is our tentative suggestion that the acetic acid-urea precipitation technique somehow manages to more exactly preserve

the native configuration of the RNA as it exists within the ribosome than does the traditional phenol extraction procedure. We explored this hypothesis by extracting 16S RNA with the acetic acid-urea technique from two different 30S ribosome preparations which have been shown by others to exist in distinctly different conformations. When ribosomes are depleted either for monovalent or for divalent cations, they undergo a significant alteration in conformation^{20,21}. The ion-depleted ribosomes are inactive for transfer-RNA binding at 0°C, but can be readily reactivated by heating for short periods in buffers containing both monovalent and divalent cations (buffer II). This reversible conformational change has been shown to involve a physical conformational change.

Figure 7 Polyacrylamide gel electrophoresis patterns of proteins tested for binding to 16S RNA extracted from two different preparations of 30S ribosomes. The RNA was purified by the acetic acid-urea procedure from 30S particles depleted of monovalent cations (gel a) and 30S subunits heat activated in the presence of monovalent and divalent cations (gel b). The mixture of proteins used to examine protein binding consisted of an equimolar mixture of proteins S3,S5,S9,S12,S13 and S18.

Thus we extracted 16S RNA, using the acetic acid-urea technique, from ion-activated 30S ribosomes and from monovalent ion inactivated particles. The resultant RNA was tested for its capacity to bind a mixture of ribosomal proteins S3,S5,S9,S12, S13 and S18. Figure 7 shows the polyacrylamide gel patterns of protein extracted from the two binding complexes. Apparently the RNA extracted from the monovalent ion depleted is far more open in configuration than the RNA extracted from ion activated 30S ribosomes. In experiments not shown, phenol extraction of 16S RNA from the two preparations of 30S particles yielded RNA totally incapable of binding this mixture of proteins. We conclude that somehow the acetic acid-urea approach to RNA purification more accurately preserves the native structure of the RNA as it exists in a given preparation of ribosomes. Furthermore, this is the first evidence that the 30S ribosome conformational change studied here involves a change in the configuration of the RNA moiety as well as the protein moiety²¹.

The Degree of Secondary Structure of 16S RNA is not Affected by the Extraction Procedure

We have shown that acetic acid-urea 16S RNA possesses a structure significantly different than 16S RNA prepared by the classical phenol procedure. Our experiments have indicated also that this difference in configuration is subtle in nature. We have attempted to determine whether the structural variants is due to a change in the number of nucleotide base pairs, which would primarily affect the secondary structure of the RNA, although the tertiary structure is affected to some degree, too.

Figure 8 is an illustration of the temperature melting profiles obtained for acetic acid-urea 16S RNA and phenol 16S RNA. In buffer II, the two types of RNA preparations have identical temperature denaturation profiles. We conclude that the two types of RNA preparations probably contain identical secondary structures. If this suggestion is true, then the structural differences we have observed between the two preparations must be due to important tertiary variations in configuration.

Figure 8 The hyperchromic effect on the absorbance at 260 nm due to the thermal denaturation of the RNA. The experiment was conducted in buffer II. o——o acetic acid-urea 16S RNA; o-----o phenol 16S RNA. The relative A_{260} is defined as A_{260} at temperature X/ A_{260} at 25°C.

There is No Difference in Primary Sequence Between the Two Preparations of 16S RNA

Although the bulk of the information presented above indicates strongly that acetic acid-urea 16S RNA and phenol 16S RNA differ only in 3-dimensional conformation, the possibility does exist that the two types of RNA instead have different lengths. We examined this postulate by conducting a 2-dimensional separation of the oligonucleotides produced by a complete T_1 -RNase and alkaline phosphatase digestion of ^{32}p labeled 16S RNA¹⁸. Figure 9 shows the results which indicate that acetic acid-urea and phenol 16S RNA most likely contain identical primary structures. Besides a few weak additional spots in the phenol 16S RNA digest, the oligonucleotide fingerprints are the same for both RNA preparations. This result strengthens out contention that the two types of RNA differ in tertiary conformation.

Figure 9 Two Dimensional Fingerprint Analysis of Acetic Acid-Urea 16S RNA and Phenol 16S RNA. The two types of RNA preparations appear to have identical fingerprints indicating no significant difference exists between their primary sequences.

DISCUSSION

We had previously documented that 16S RNA extracted by a mixture of urea and acetic acid, is capable of binding specifically 13 ribosomal proteins¹. In contrast 16S RNA prepared by the traditional phenol method yields RNA containing only 7 protein binding sites²⁻⁷. The most direct explanation of these differences in protein binding capacity of the two types of RNA is that they possess substantially different 3-dimensional structures. We have examined in this communication the conformation of acetic acid-urea 16S RNA and of phenol 16S RNA by a number of experimental criteria.

Two of the conformational parameters tested showed no significant difference between acetic acid-urea and phenol 16S RNA. The two preparations of RNA have identical sedimentation properties and show exactly the same temperature denaturation characteristics. Furthermore, 2-dimensional fingerprint analysis of ³²p labeled RNA indicates that the technique used in the RNA preparations does not alter the primary sequence of the RNA.

These observations suggest that the two types of RNA do not differ either in primary structure or secondary structure.

Acetic acid-urea 16S RNA, however, is significantly more susceptible to T_1 -RNase attack than is phenol 16S RNA. The kinetics of T_1 -RNase digestion at 4°C imply that the RNA molecules prepared by the acetic acid-urea procedure are considerably more extended, open to nucleolytic attack, than are the phenol RNA molecules. The proposed relative more "open configuration" of acetic acid-urea RNA is corroborated by a polyacrylamide gel electrophoretic analysis of the limited T_1 -RNase digestion products. At the early stages in the digestion process, phenol 16S RNA shows numerous high molecular weight fragments which are not present in similar samples of acetic acid-urea 16S RNA.

Perhaps more convincing evidence that the two preparations of 16S RNA have different 3-dimensional configurations resides in our observation that they exhibit measurably distinct electrophoretic mobilities, using an agarose-polyacrylamide composite gel system reputedly sensitive to RNA conformation¹⁸. In this gel electrophoresis system acetic acid-urea 16S RNA migrates more slowly than does the 16S RNA prepared by the phenol technique. This result, taken together with the greater nuclease sensitivity of acetic acid-urea RNA, strongly suggests that phenol RNA must exist in a relatively more restricted and "closed conformation". Thus it seems likely that there is a valid correlation between the open conformation maintained by acetic acid-urea 16S RNA and its capacity to individually bind 13 ribosomal proteins.

This conclusion is strengthened by our observation that the open configuration of acetic acid-urea 16S RNA is unstable and can be transformed by storage into a conformation which closely resembles that possessed by phenol 16S RNA. Concomitant with this change in conformation from an open structure into a closed structure, as detected by agarose-polyacrylamide gel electrophoresis, the acetic acid-urea RNA gradually loses its ability to bind all 6 new proteins. Storage of this RNA ultimately transforms it into a preparation which can bind only 7 ribosomal proteins, identical to phenol 16S RNA.

Figure 10 A schematic summary of our proposed role of the two different 16S RNA conformations in the mechanism of *in vitro* assembly and ribosome structure.

All of our results can be summarized schematically as illustrated in Figure 10. Our experiments have shown that 16S RNA can exist in two different and readily distinguishable conformations. In Figure 10 we refer to these conformations as the "open configuration" (acetic acid-urea 16S RNA) and the "closed configuration" (phenol preparation). As this figure also illustrates, we have shown that the acetic acid-urea extraction procedure to some extent maintains the conformation of 16S RNA as it exists within the intact ribosome. The open configuration can only be derived from cation depleted 30S ribosomes, whereas the closed configuration is assumed by RNA molecules extracted from cation activated ribosomes.

These observations lead to an apparent paradox. The RNA as it exists in cation activated 30S particles seems to be in a closed conformation, exhibiting considerably fewer specific protein binding sites. This apparent paradox can be rationalized by assuming that the open configuration exists only during certain stages of 30S *in vitro* ribosome assembly. We postulate that the proteins which have been shown to bind during early phases of the assembly sequence² (i.e. proteins S4, S7, S8, S15,

S20) transform the closed configuration into the open configuration allowing proteins S3, S5, S9, S11, S12 and S18 to participate in the formation of the complex. Indeed, in preliminary experiments we have shown that the closed configuration undergoes an expansion into a form identical in electrophoretic mobility to the open form after binding a set of these proteins.

In order to explain the re-emergence of the closed configuration in cation activated 30S ribosomes, we propose that the RNA undergoes a second conformational change at some later stage in ribosome assembly. Experiments are presently being conducted which challenge these speculations.

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