Detection of cation-specific conformational changes in ribosomal RNA by gel electrophoresis

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ABSTRACT

Electrophoresis of ribosomal RNA in polyacrylamide-agarose composite gels separates 16S and 23S species into multiple bands. These bands of RNA represent multiple conformational forms of the molecules as judged by oligonucleotide analysis of the 16S RNA. Gel electrophoresis was used to test for cation-specific conformational changes in ribosomal RNA. Relative to magnesium-equilibrated RNA, barium ion and putrescine induced alterations in the electrophoretic behavior of ribosomal RNA while calcium ion produced no change. Exchange of a critical level of bound magnesium ion for barium or putrescine was necessary for these changes to take place. The alterations in electrophoretic behavior were unaffected by simply restoring magnesium ion, but in addition required heating for reversal. We suggest that these conformational changes are a result of interaction at a specific class of cation binding sites previously observed with intact ribosomes.

INTRODUCTION

Previous studies suggested that ribosomes of *Escherichia coli* contained at least three classes of cation binding sites (1,2,3). These sites were defined by the specificity for particular cations in the maintenance of intact, functioning ribosomes. Occupation of sites by inappropriate cations led to conformational changes and inactivation. It was suggested that the specificity of these sites arose from structural limitations on the interaction of cations with RNA in the native ribosomal particle. The present study was undertaken to see if such specificity could be detected in the interaction of cations with isolated ribosomal RNA.

Electrophoresis of ribosomal RNA in agarose-acrylamide gels displays multiple bands of the 16S and 23S species, which are thought to represent different conformational states of the molecules (4,5). In the work reported here, we confirm that these multiple bands represent conformational isomers of the RNAs since, at least in the case of 16S RNA, all forms contain the normal 5' and 3' oligonucleotides. Using gel electrophoresis, we detect multiple forms of ribosomal RNAs which differ from each other

because of bound cations. We find that cation specificity for interconversion of these forms correlates with the specificity which has been demonstrated for intact ribosomes.

METHODS

Ribosomes were prepared (1) and RNA extracted (2) as previously described. After precipitation of the RNA with ethanol, it was resuspended at a concentration of approximately 10 mg/ml in a buffer containing 10 mM Tris-HCl (pH 7.5), 60 mM KCl and 10 mM $MgCl_2$. After overnight dialysis against the same buffer, the RNA preparations were stored at -80° .

Equilibrium dialysis and determination of bound magnesium by atomic absorption spectroscopy were carried out as described previously (1). In general, dialysis was performed with an RNA concentration of 2 mg/ml for 12 hours at 3° . The buffer employed was the Tris-KCl buffer described above with 1 mM of the appropriate cation chloride salt replacing $MgCl_2$.

The techniques for gel electrophoresis were similar to those previously employed (4,5). Composite slab gels consisting of 3% acrylamide and 0.5% agarose were constructed in the electrophoretic cell supplied by E. C. Apparatus Corp., St. Petersburg, Florida. Electrophoresis was carried out for 7.5 hours at 200 volts and 0° in Tris-EDTA-borate buffer, pH 8.3. After electrophoresis the gels were stained with "Stains-All" (6) and either photographed or scanned in a Joyce Loebel microdensitometer.

For oligonucleotide analysis of 16S RNA, cells of strain CP78 were grown in 10 ml of defined medium (7) containing $0.2 \text{ mCi/ml } ^{32}PO_4^{3-}$ for one hour at $37^\circ C$. The cells were chilled on ice and then spun out of the medium before being frozen in a dry-ice ethanol bath. RNA was prepared and purified by electrophoresis as described elsewhere (4,5).

Radioactive 16S RNA was located on the gel by autoradiography. The regions of the gel containing the rapidly and slowly migrating bands of 16S RNA were cut out and mascerated by squeezing between two glass slides. RNA was eluted by shaking the gel for 12 hours in 3 ml 0.3 M NaCl. 100 μg m carrier RNA was added and the gel was removed by filtration through nitrocellulose (0.45 μ) filters. Two volumes ethanol were added to the filtrate. After standing at $-20^\circ C$ overnight, the precipitate was collected, resuspended in 2 ml 0.2 M sodium acetate at $0-5^\circ C$, refiltered and reprecipitated with ethanol. This resuspension and refiltration was repeated two more times, to remove eluted agarose. The resulting RNA was sufficiently clean to be resuspended in 5 μl RNase T_1 plus alkaline phosphatase as described

Figure 1. Electrophoresis of ribosomal RNA extracted in the absence of multivalent cations. Whole cell RNA was extracted and electrophoresed as previously described (4,5). Figure 1A is a photograph of the stained gel and m16 and m23 refer to the mature species of 16S and 23S ribosomal RNA. Figure 1B is a densitometric scan of the stained gel.

Figure 2. Oligonucleotides of rapidly and slowly migrating bands of m16S RNA. The RNA was labeled with $^{32}\text{PO}_4^{3-}$, purified by gel electrophoresis, eluted from the gel and digested with RNase T₁ and alkaline phosphatase as described in Methods and elsewhere (4-9). Oligonucleotides arising from the 5' and 3' ends of the mature 16S RNA (10) are indicated. **A.** Oligonucleotides produced by digestion of rapidly migrating m16 RNA (cf. band A of m16 in Figure 1A). **B.** Oligonucleotides produced by digestion of slowly migrating m16 RNA (cf. band C of m16 in Figure 1A).

by Sanger (8,9). Recovery of 16S RNA from the gel by this method was about 25-30 per cent efficient. The RNA was digested and the oligonucleotides were analyzed according to the methods of Sanger (8,9).

RESULTS

When RNA was extracted from whole cells into a buffer lacking multivalent cations, the electrophoretic pattern pictured in Figure 1A was obtained. This pattern displays the multiple bands of the mature ribosomal RNA species reported previously (4,5). The 16S region separated into three bands of equal intensity (labeled A, B, and C) and the 23S region displayed two bands (A and B), also of equal intensity. A densitometric scan of the gel is shown in Figure 1B.

In order to determine whether the multiple bands of 16S RNA result from populations of molecules which differ in length, the fastest and slowest migrating forms were eluted from the gel and analyzed by RNase T₁ plus alkaline phosphatase digestion and fingerprinting. The patterns of oligonucleotides obtained are shown in Figures 2A and 2B. Careful inspection of the oligonucleotide fingerprints revealed no differences between the RNAs of the two preparations. In particular, both species contained the 5' and 3' terminal oligonucleotides (10), indicated by the arrows on the figures. The compositions of those terminal oligonucleotides were confirmed by elution from the paper and redigestion with pancreatic RNase (7-9). We conclude that the multiple forms of mature 16S RNA which can be separated on these gels probably reflect differences in conformation rather than in chain length.

The influence of equilibration of RNA with various divalent cations on the conformation of the RNA was investigated (Figure 3). Scan 1 illustrates the electrophoretic behavior of magnesium-equilibrated RNA. The pattern in the 23S region was essentially the same as that obtained in the absence of multivalent cations (Figure 1), band A being approximately equal in intensity to band B. However, in the 16S region distinct differences were observed. Whereas in the absence of multivalent cations bands A, B, and C were of roughly equal intensity, with magnesium-equilibrated RNA band C was essentially absent and the intensity of band A was considerably greater than that of band B (Scan 1). When greater than 95% of the bound magnesium ion was replaced by dialysis against 1 mM calcium ion, no significant changes in the electrophoretic pattern were observed (Figure 3, Scan 2). In contrast, when magnesium ions were displaced by 1 mM concentrations of

Figure 3. Electrophoresis of ribosomal RNA after equilibration with various divalent cations. Ribosomal RNA was dialyzed to equilibrium against Tris-KCl buffer containing: Scan 1, 1 mM $MgCl_2$; Scan 2, 1 mM $CaCl_2$; Scan 3, 1 mM $BaCl_2$; and Scan 4, putrescine $\cdot$ 2 HCl. In the latter three instances, the ratio of remaining bound magnesium to RNA phosphate was less than 0.01. Densitometer tracings of the stained gels are shown.

barium ions or the organic cation putrescine, distinct changes in electrophoretic behavior were observed. With barium ions, there was a definite shift toward the slower migrating species in both the 23S and 16S regions (Figure 3, Scan 3). With putrescine (Figure 3, Scan 4), the 23S RNA showed the same tendency toward slower moving species as was observed with barium ions, only to a lesser extent. Putrescine produced no significant difference in the mobility of 16S RNA from the magnesium-equilibrated control. If the concentration of barium ions or putrescine in the dialysis buffer was 1 mM, but sufficient magnesium ions were present to maintain the ratio of bound magnesium to RNA phosphate above 0.12, the electrophoretic patterns remained identical to the magnesium control (data not shown). Thus, these electrophoretic changes were not due simply to the presence of barium ions or putrescine, but required displacement of a critical level of magnesium ions. These results were not dependent upon the method of preparation of the RNA, since RNA prepared by the urea-LiCl procedure of Nomura and Erdmann (11) behaved identically to the RNA shown here.

The reversibility of these alterations in electrophoretic behavior of RNA was next investigated. The ribosomal RNA pattern produced by barium ions (Figure 4) was not reversed by dialyzing the preparation at 3° against a buffer containing 20 mM magnesium ions (compare Scans 2 and 3). However, when the preparation in 20 mM magnesium ions was heated for 5 minutes at 40°, the original electrophoretic pattern was restored (Scan 4). Similar results were obtained with RNA preparations in which magnesium ions had been replaced by putrescine (Figure 5). Simply restoring the RNA to a buffer containing 20 mM magnesium ions without heating did not reverse the putrescine-induced changes in the 23S RNA molecule. These results argue against the possibility that the slowed electrophoretic migration of the RNA in the presence of barium ions or putrescine was due to the higher atomic or molecular weight of the counter ions, since reversal of these effects required heating in addition to replacement with magnesium ions.

DISCUSSION

Composite gel electrophoresis resolves ribosomal RNA into multiple bands (4,5). Our fingerprint analysis of the RNAs isolated from these bands indicates that the bands result from conformational rather than primary sequence differences. The electrophoretic conditions used here (zero degrees and an EDTA-containing buffer) are rather stringent and most

Figure 4. Reversibility of the changes in electrophoretic behavior induced by barium ion. Scans 1 and 2 were obtained with magnesium- and barium-equilibrated RNA as in Figure 3. A sample of the RNA shown in Scan 2 was dialyzed overnight against a buffer containing 5×10^{-3} M (K)phosphate (pH 7.8), 20 mM MgCl_2 , and 0.3 M KCl, kept at 3° and electrophoresed (Scan 3). These conditions of dialysis restore the original level of bound magnesium and should have resulted in removal of >95% of the barium ion in this experiment or of putrescine in Figure 5 (1,3). A portion of the RNA from Scan 3 was heated for 5 minutes at 40° prior to electrophoresis (Scan 4). Dialysis of the RNA displayed in Scan 1 against the phosphate- MgCl_2 -KCl buffer showed no influence on the electrophoretic pattern, with or without heating at 40° .

Figure 5. Reversibility of putrescine-induced alterations in electrophoretic behavior. This experiment was carried out identically to that of Figure 4, except using putrescine rather than barium ion. Scans 1 and 2 represent magnesium- and putrescine-equilibrated RNA respectively. The putrescine-equilibrated RNA was dialyzed against the phosphate-MgCl₂-KCl buffer (Figure 4) and electrophoresed without (Scan 3) or with (Scan 4) heating at 40° for 5 minutes.

likely accentuate conformational differences by "locking" the ribosomal RNA into specific structures. These conditions are essential, however, to detect differences in RNA which otherwise go unresolved at higher temperatures (4,5) or in a magnesium-containing buffer (6).

The cation-specific changes in conformational isomers of ribosomal RNA we report here correlate well with similar cation-specific changes reported previously for ribosomes (1-3). However, the RNA conformations observed in the gel cannot necessarily be equated to the precise conformations of RNA within the ribosome or free in solution. Nor are these conformation differences necessarily related to those reported by Loening during a study of changes in composition of the electrophoresis buffer (12).

As mentioned in the Introduction, three classes of cation binding sites have been defined on intact ribosomes of *E. coli* (1-3). The Class I sites (approximately 20% of the total) must be filled by magnesium ion, or other multivalent cations with crystalline ionic radii less than 1 Å, to preserve structure and function of the particles. The Class II sites (also approximately 20% of the total) must be neutralized by multivalent cations as well, but these sites are considerably less specific, with any multivalent inorganic or organic cation sufficing. The remaining 60% of the ribosomal cation binding sites (Class III) appear to be totally non-specific, since neutralization by any monovalent or multivalent cation is sufficient. The cation-specific conformational changes reported here with isolated ribosomal RNA resemble the characteristics of the Class I sites of intact ribosomes in several respects. First, the molar ratio of bound magnesium to RNA phosphate must be reduced below 0.12 in order to observe the conformational changes in isolated RNA. This was also one of the distinguishing characteristics of the Class I sites in intact particles. A second common characteristic is that the conformational changes could not be reversed by simple restoration of magnesium ions, but heating was also required. The third argument that the conformational changes observed in isolated RNA and those in ribosomes are facets of the same phenomenon lies in the cation specificity. With intact ribosomes, filling the Class I sites with barium ions (3) or putrescine (1,2), led to conformational changes and inactivation of both the 30S and 50S subunits. In contrast, calcium ions were fully sufficient for maintenance of the structure of the 30S subunit and partially effective for the 50S particle (3). Thus, the cation specificity of the structural changes in ribosomal RNA reported here was similar to the specificity of the Class I cation binding sites defined

with intact ribosomal particles. The similarities between results obtained with isolated ribosomal RNA and intact particles support the previous conclusion that the cation specificities observed with ribosomes were a result of cation-specific binding sites on the ribosomal RNA (3). However, we recognize that the data do not prove that the particle changes can all be accounted for by changes in the RNA alone.

It seems clear from these studies as well as those of others that naturally occurring RNA molecules contain specific cation binding sites and that variation in the neutralizing counter ion can produce alternate molecular forms of the RNA molecule. Studies of divalent cation binding by transfer RNA and ribosomal RNA suggested the presence of a small class of strong, cooperative binding sites (13-15). In addition, both the biological and physical characteristics of transfer RNA and 5S ribosomal RNA are dependent on the nature of the interacting counter ions (16-21). Sypherd reported conformational changes in 16S RNA during reconstitution of the 30S subunit which may be related to these phenomena (22). The exact nature of this cation specificity in terms of molecular interactions is purely a matter of conjecture at present. It may be that cation size, charge density or degree of hydration all influence interaction between the ions and various types of secondary and tertiary structures in the RNA.

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