Molecular Integrity of Plant Ribosomal Ribonucleic Acid

ANGUS G. HEPBURN1 AND JOHN INGLE
Department of Botany, University of Edinburgh, Edinburgh, EH9 3JH, Scotland

Received for publication June 17, 1975 and in revised form October 10, 1975

ABSTRACT

Thermal denaturation of plant ribosomal RNA followed by gel fractionation shows that although a large percentage of molecules contain breaks in the polynucleotide chain, 25S and 18S RNAs do exist as unique molecular species. Values for the rate constant of hydrolysis under routine denaturing conditions are of the order of $10^{-2}$ to $10^{-4}$ sec$^{-1}$ and these are shown not to be a result of ribonuclease activity. This high rate of hydrolysis and the use of insensitive fractionation procedures may account for the reported absence of a 25S rRNA molecule and its apparent conversion to a molecule similar in size to 18S RNA.

Shine and Dalgarno (17) showed by denaturation studies that the native 26S rRNA molecule from a range of insects comprises two molecules of RNA approximately equal in weight, and a third of low mol wt (the 7S RNA) (14). The size of the two major molecules is similar to that of the 18S rRNA molecule, so close that in some cases they co-sediment or co-electrophorese (1). Attempts have been made to determine whether a similar situation holds for the 28S rRNA in mammals (16) or reptiles (5), the 23S rRNA in bacteria (18), and the 25S rRNA in plants (7, 21), using a range of different techniques. With the two plant species, it was claimed that pea (7) and soybean (21) 25S rRNA was converted to an 18S product by denaturation, indicating that it did not exist as a covalently bonded molecule. Apart from considerations of molecular integrity, the actual sizes of rRNA molecules as determined by gel electrophoresis of native preparations has also been called into question. Reijnders et al. (15) showed considerable differences between the sizes obtained for molecules of rRNA in the native and denatured state, presumably as a result of differences in the secondary structure of the rRNA from different species.

We show in this report that the 25S rRNA from two plant species and the 28S rRNA from mouse liver are true molecular species and that at least some of the reports of conversion of these to 16 to 18S products by denaturation are a direct result of excessively severe denaturation conditions causing hydrolysis of the phosphodiester bonds, coupled with the insensitivity of fractionation by sucrose density gradient centrifugation. To determine the degradative effect of denaturation, the rate constant for ribonucleotide phosphodiester bond hydrolysis was measured under a range of routine denaturation conditions. We also show that, as with animal rRNA, the apparent mol wt of plant rRNA in the native and denatured state are different when compared to Escherichia coli rRNA, but that the differences for plants are not of the same magnitude as those for animals.

MATERIALS AND METHODS

Escherichia coli cells were harvested after 24 to 36 hr of incubation at 37°C in a medium containing 10 g/l nutrient broth, 5 g/l yeast extract, 2.5 g/l glucose, and 5 g/l NaCl, adjusted to a final pH of 7.5, and lysed in 50 mM NaCl, 10 mM tris-HCl (pH 7.4), containing 6% PAS, and 1% TNS together with enough phenol mixture (phenol saturated with 10 mM tris-HCl, pH 7.4, containing 10% m-cresol and 0.1% 8-hydroxyquinoline) to dissolve the PAS.

Whole livers from freshly killed mice were chilled in ice and homogenized in a VirTis homogenizer in 50 mM NaCl, 10 mM tris-HCl (pH 7.4). PAS and TNS were added to a final concentration of 6 and 1%, respectively, together with a small amount of phenol mixture.

Pea epicotyls were harvested after 3 to 5 days of dark growth at 25°C and ground in a mortar and pestle in the same detergent medium as that used for lysing E. coli cells. Artichoke tuber explants, excised and cultured for 3 days as described by Yeoman and Davidson (20) were similarly homogenized in detergent medium.

Total nucleic acid was prepared from these initial homogenates as described by Leaver and Ingle (9), the standard Kirby method. Purification of rRNA was achieved by the 2.7 M NaCl precipitation technique of Parish and Kirby (12).

The technique for polyacrylamide gel electrophoresis was essentially that described by Loening (10). For electrophoresis under denaturing conditions, the acrylamide concentration was reduced to 2%, and the gels were polymerized in the presence of 7 M urea. The electrophoresis buffer (buffer E: 30 mM NaH2PO4, 1 mM EDTA, 36 mM trisma base, final pH 7.6 to 7.8, containing 0.2% SDS) was also made 7 M with respect to urea (buffer UE) and the RNA fractionated at 50°C. The gels were scanned in a Joyce-Loebl UV scanner and the $E_{260}$ continuously recorded.

Since the denatured RNA samples were to be immediately analyzed by gel electrophoresis, all denaturations were done in buffers E or UE containing 12% sucrose. The hyperchromicity curves were obtained for RNA preparations in the above buffers in a Pye Unicam SP 800 recording spectrophotometer with the sealed cells containing the samples placed in a temperature-controlled block. The temperature was increased 1°C per 3 min, and the temperature and absorbance at 265 nm were recorded every minute.

RESULTS

The thermal denaturation profiles of pea rRNA in buffers E and UE (Fig. 1) shows that while a temperature of 90°C is required to produce maximum hyperchromicity in buffer E, the presence of 7 M urea in the incubation mixture reduces this temperature to 50 to 60°C. In order to determine whether

---

1 Present address: Department of Botany, University of Georgia, Athens, Ga. 30602.

2 Abbreviations: PAS: p-aminosalicylic acid-Na salt; TNS: trisopropynaphthalene sulphonic acid-Na salt.
fractionation under denaturing conditions was necessary for the analysis of denatured samples, or whether denaturation followed by normal fractionation would suffice. *E. coli* rRNA was analyzed by these two methods. When *E. coli* rRNA, purified by NaCl precipitation (12) (23S to 16S ratio of 2.83), was denatured for 15 min, buffer E gel fractionation at room temperature showed that there was a 45% loss of the 23S and a 30% loss of the 16S molecules which reduced the 23 to 16S ratio to 2.23 (compare Fig. 2, a and c). When the denatured rRNA was fractionated on buffer UE gels at 50 °C for 3 hr, there was further loss of the two major components, the ratio decreased to 2.00 and tails were visible on the low mol wt side of the two peaks (Fig. 2b). Incubation for 3 hr 15 min in buffer UE at 50 °C followed by buffer E gel fractionation at room temperature resulted in virtually identical recovery of the two major components as that obtained when the RNA was fractionated at 50 °C in UE gels after a 15-min denaturation, with the 23S to 16S ratio reduced to 2.04 (Fig. 2d). The presence of the tails on the UE buffer gel fractionation profile is caused by molecules being hydrolyzed during fractionation and is not indicative of the presence of fragments of rRNA in the sample with these mobilities. When the denaturation and hydrolysis are completed prior to fractionation, the fragments appear as an overall raising of the baseline of the profile from the peak of 23S to the low mol wt end of the gel (Fig. 2d). Thus in terms of the analysis of intact molecules, incubation prior to fractionation is sufficient to ensure that complete denaturation has been achieved.

The effect of incubation on rRNA at elevated temperatures is 2-fold. During the first 5 min of denaturation, the amount of intact plant 25 and 18S rRNA decreases by 40 to 60% depending on the preparation used, values rather larger than those obtained for *E. coli* rRNA. Thereafter, the amount of the two intact species decreases to zero over a period of hours. The first part of this decrease shows no obvious kinetics and presumably represents the release of fragments from molecules containing breaks, occurring as the regions of secondary structure, which initially hold the molecule together are denatured. The second phase of the loss of the 25 and 18S components shows true first order (or pseudo-first order since water and possibly urea are present in excess) reaction kinetics with a linear decrease in the
by two successive elutions in 1 ml 1 M lithium acetate, 0.5% SDS at 25 C, the first for 14 hr, the second for 5 hr, and collected by centrifugation of 100,000 g for 17 hr. An adaptation of the procedure of Cox (3) was used whereby the complete preparation in 50 mM NaCl, 10 mM Tris-HCl (pH 7.4) was made 4 M with respect to guanidinium chloride (BDH, Aristar grade) and, after the addition of 0.5 volume of ethanol, precipitated overnight at 0 C. The precipitate was collected by low speed centrifugation, washed with 80% aqueous ethanol, 0.2% SDS, and repurified from 150 mM sodium acetate, 0.5% SDS by the addition of 2 volumes of ethanol. The values of kₕ, determined under the conditions of 60 C in buffer UE for the original and the two further purified samples were identical (Table IB), indicating that the observed hydrolysis was not due to residual ribonuclease activity in preparations made by the standard Kirby method.

Implicit in the above work is the observation that after incubations under conditions which completely denatured rRNA, intact rRNA molecules were still observed on polyacrylamide gel profiles. That these apparently intact molecules were not the result of reassociation of fragments between the end of the denaturation treatment and the point at which the electrophoretic process had spatially separated the complementary fragments was shown by following 5-min denaturations by incubation at 25 C for periods up to 1 hr. The fractionation profiles of these samples showed no increase in the amount of the two mature rRNA components with time.

Figure 3 shows the effect of 5-min incubations at different temperatures on the 2.4% polyacrylamide gel electrophoretic profiles of pea total nucleic acid. Fractionation of the untreated sample resolves the DNA, the two rRNAs with mol wt of 1.27 and 0.69 x 10⁶ plus small peaks of the organelle RNAs at 1 and 0.5 x 10⁶ daltons and a trace of 0.69 x 10⁶ aggregate at 1.4 x 10⁶ daltons (Fig. 3a). Incubation for 5 min at 30 C in E buffer does not alter this profile (Fig. 3b), but after 5 min at 50 C (Fig. 3c) there is considerable loss of the 1.27 and 0.69 x 10⁶ mol wt rRNA components (relative to the DNA) and the former is reduced in size to 1.22 x 10⁶ daltons by the loss of the 7S component (14). Many additional peaks are present at 0.32, 0.43, 0.58, 0.96, and 1.80 x 10⁶ daltons, the latter being an aggregate peak of 25S and 18S RNA, and there is a background of heterogeneous molecules with a mean at about 1.3 x 10⁶ daltons. Increasing the incubation temperature removes the aggregate peak (Fig. 3d) and progressively reduces the mean of the heterogeneous background to 0.4 x 10⁶ daltons (Fig. 3e). Comparable treatment in buffer UE results in a similar degree of degradation being achieved at a lower temperature. For example, treatment at 30 C in buffer UE (Fig. 3f) gives a profile similar to that resulting from exposure to buffer E at 50 C except that the presence of urea greatly reduces aggregation. After treatment in buffer UE at 70 C, there are still traces of the intact rRNA molecules on a high heterogeneous background with a mean of about 0.5 x 10⁶ daltons (Fig. 3h), whereas exposure to

### Table I. Rate of Phosphodiester Bond Hydrolysis (kₕ) for rRNAs

Purified rRNA prepared by the standard Kirby method was incubated as shown and fractionated on either 2.4% polyacrylamide gel E gels at room temperature or 2% polyacrylamide gel buffer UE gels at 50 C. The concentration of intact rRNA molecules was determined from the gel profiles and plotted against time. The rate constant for hydrolysis of the RNA (kₕ) was calculated from the slope. This was converted to the rate constant for phosphodiester bond hydrolysis (kₕ) by dividing by the number of bonds per molecule. Samples hydrolyzed at 50 C in buffer E were made 7 M with respect to urea and incubated for a further 5 min immediately prior to electrophoresis to ensure complete denaturation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Hydrolysis Conditions</th>
<th>25/28S rRNA</th>
<th>16/18S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli (1.1 and 0.56 x 10⁶ daltons)</td>
<td>50 C, buffer UE</td>
<td>1.4 x 10⁻⁶</td>
<td>1.8 x 10⁻⁶</td>
</tr>
<tr>
<td>Artichoke (1.3 and 0.7 x 10⁶ daltons)</td>
<td>50 C, buffer UE</td>
<td>4.04 x 10⁻⁴</td>
<td>3.49 x 10⁻⁴</td>
</tr>
<tr>
<td>Mouse (1.78 and 0.7 x 10⁶ daltons)</td>
<td>50 C, buffer UE</td>
<td>1.4 x 10⁻⁴</td>
<td>3.70 x 10⁻⁴</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.4 x 10⁻⁴</td>
<td>2.39 x 10⁻⁴</td>
</tr>
<tr>
<td>Mouse</td>
<td>50 C, buffer E</td>
<td>6.09 x 10⁻⁷</td>
<td>9.40 x 10⁻⁷</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.58 x 10⁻⁷</td>
<td>7.75 x 10⁻⁷</td>
</tr>
</tbody>
</table>

### Table II. Effect of Further Purification of RNA on kₕ at 60 C in Buffer UE

Pea rRNA was prepared by the standard Kirby method and then further purified as described in the text and incubated at 60 C in buffer UE for increasing periods of time and fractionated on 2.4% polyacrylamide gel E gels at room temperature. The values of kₕ and the derived kₕ were calculated as described in Figure 1a.

<table>
<thead>
<tr>
<th>Purification Method</th>
<th>25S rRNA kₕ (sec⁻¹)</th>
<th>18S rRNA kₕ (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Kirby</td>
<td>2.44 x 10⁻⁷</td>
<td>2.70 x 10⁻⁷</td>
</tr>
<tr>
<td>Electrophoretically purified</td>
<td>2.47 x 10⁻⁷</td>
<td>2.29 x 10⁻⁷</td>
</tr>
<tr>
<td>Guanidinium chloride precipitated</td>
<td>2.50 x 10⁻⁷</td>
<td>2.51 x 10⁻⁷</td>
</tr>
<tr>
<td>Mean kₕ</td>
<td></td>
<td>2.49 x 10⁻⁷</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of temperature on the stability of pea nucleic acid. The profiles of 2.4% polyacrylamide gel E gels fractionated for 2.5 hr at room temperature show native RNA (a) and samples incubated for 5 min in either buffer E at 30 C (b), 50 C (c), 70 C (d), and 90 C (e), or buffer UE at 30 C (f), 50 C (g), 70 C (h), and 90 C (i). Both buffers contained 12% sucrose, and the samples were rapidly cooled in ice prior to fractionation.
90 C removes all traces of discrete peaks resulting in a heterogeneous distribution with an average size of 0.1 to 0.2 \times 10^6 daltons (Fig. 3i). Thus if denaturation is measured in terms of the release of discrete fragments rather than by increase in hyperchormicity, the minimum temperatures required are 50 C in buffer E and 30 C in buffer UE. Slightly higher temperatures are required in buffer E to remove the aggregation, which may however, be prevented by reducing the RNA concentration in the incubation mixture from 1 mg/ml to less than 500 \mu g/ml. The continued loss of the major rRNA components as the temperature in buffer UE is increased above 60 C, which according to the hyperchormicity curve in Figure 1 should produce complete denaturation, is a reflection of the increasing rate of thermal hydrolysis. Thus at temperatures where denaturation is complete, intact 25S and 18S rRNA molecules persist. A similar persistence of intact 25S and 18S rRNA was observed after incubation for 5 min at 60 C with an equal volume of buffer-saturated phenol (Fig. 4) which, it has been previously claimed, converts pea 25S rRNA to a 17 to 18S molecular species (7).

In order to determine the effect of secondary structure on the apparent mol wt of rRNA, the relative mobilities of rRNA from a range of organisms was determined with respect to E. coli rRNA (1.1 and 0.56 \times 10^6 daltons [11]) in the native and denatured states. Table II shows the mol wt obtained. The plant 18S rRNA showed a similar small change (3%) in apparent mol wt as that found for mouse liver 18S rRNA (4%). While the mouse 28S rRNA showed an apparent drop in mol wt of 20% after the removal of all secondary structure, the mol wt of artichoke 25S rRNA dropped by only 6% and that of pea 25S rRNA increased by 4%. Thus in these two plant species, the denaturation produces only small changes in the electrophoretic mobility of rRNA relative to E. coli rRNA as compared with the large change observed with the mammalian RNA examined, a result similar to that obtained by Reijnders et al. (15).

**DISCUSSION**

The values reported here for the rate constant of phosphodieser bond hydrolysis compare favorably with those reported elsewhere for the thermal hydrolysis of other phosphate linked compounds, e.g. dimethyl phosphate at 100 C (1 \times 10^{-9} sec^{-1}; [2]) and dibenzyl phosphate at 75 C (4 \times 10^{-7} sec^{-1}; [8]). The rate constant is also similar to that determined for TMV-RNA in 10 mm phosphate buffer (pH 7.1) at 57 C of 1.4 \times 10^{-8} sec^{-1} (4) (c.f. k_b in buffer E at 50 C of 7.75 \times 10^{-8} sec^{-1}, Table IA). Ginoza (4) showed that for TMV-RNA, the half-life (t_1/2) during incubation at 55 C in 0.1 m phosphate buffer (pH 7.4) was 39 min as determined by the retained infectivity of the RNA and concluded that inactivation was caused by breaking one phosphodiester bond. Assuming that there are 5900 bonds per TMV-RNA molecule, the value of k_TMV at 50 C in buffer E is 4.6 \times 10^{-8} sec^{-1} which would predict a t_1/2 of 25 min, very similar to that reported by Ginoza (4).

It is clear then that RNA in solution under denaturing temperatures has a finite low t_1/2 which could explain the data of Higo et al. (7) and Yokoyama et al. (21). The absence of recognizable rRNA molecules after a 5-min incubation at 90 C (Fig. 2i) is a reflection of the very low t_1/2 of the RNA under these conditions. Projecting from the data shown in Tables IA and IB, the k_b under these conditions would be expected to be of the order of 2.5 \times 10^{-4} sec^{-1} which gives an expected t_1/2 for pea rRNA of 7 sec for the 25S and 13 sec for the 18S molecules.

It is clear that, contrary to earlier reports, plant 25S rRNA is similar to mammalian 28S rRNA in that the native molecule comprises one large polynucleotide chain and one small polynucleotide chain, the 7S, and that these are produced from a common precursor by the final processing event in the maturation of ribosomal RNA (6).

The apparent conversion of 25S rRNA to a 17 to 18S component on denaturation is due in part to the large numbers of hidden nicks in native rRNA molecules which are exposed on denaturation, in part to excessive denaturation treatments which result in considerable hydrolysis and in part to the use of insensitive fractionation procedures such as sucrose density gradients.

The fragments released by denaturation of pea nucleic acid in buffer E at 50 C (Fig. 2c) are the same as those found when native RNA from pea microsomes is analyzed by gel electrophoresis (13), the slight differences in sizes being a reflection of the 3 to 4% difference between the sizes of pea 25 and 18S ribosomal RNA determined here and by Payne and Loening (13).

The true mol wt of the plant rRNAs determined by co-electrophoresis with E. coli rRNA under denaturing conditions show that the mol wt are very similar to those obtained for native molecules, unlike those of mammalian RNAs (15) indicating that the secondary structure of the plant RNA is more similar to E. coli rRNA than is the secondary structure of mouse rRNA.

The validity of determining mol wt from denatured rather than native rRNA molecules (15) depends on the mol wt of mammalian 28S rRNA as estimated by other means. The most recent mol wt based on electron microscopic determinations (19) suggest that HeLa 28S rRNA is at least 2.5 times larger than the
18S, supporting the mol wt of 1.7 to 1.8 x 10^6 obtained from native fractionation rather than the 1.4 to 1.5 x 10^6 obtained for fully denatured molecules. On this basis, native rRNAs appear to give a more linear relationship between the log of mol wt and electrophoretic mobility than do denatured rRNAs.

LITERATURE CITED