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Short Communications

Molecular Integrity of Chloroplast Ribosomal Ribonucleic Acid

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The majority of chloroplast 1.1×10^6-mol.wt. rRNA molecules are nicked at specific points in the polynucleotide chain, the molecules being kept intact at low temperatures by their secondary structure. Conditions that break hydrogen bonds and lead to loss of secondary structure cause dissociation of the molecule.

We have previously shown (Leaver & Ingle, 1971) that the heavier of the two high-molecular-weight chloroplast rRNA components (1.1×10^6 mol.wt.) from a variety of plants is unstable under normal conditions of extraction and fractionation. In contrast, the light chloroplast rRNA (0.56×10^6 mol.wt.) remains intact under similar conditions.

It seems likely that cleavage of the rRNA chain occurs after transcription, either in the tissue or during isolation of the RNA, as the cleavage products are prevented from separating by bivalent cations such as Mg^{2+} and Ca^{2+}, but do not associate again in the presence of these cations once they have parted (Leaver & Ingle, 1971).

In the present communication I show that the integrity of the 1.1×10^6-mol.wt. rRNA can be maintained in the absence of bivalent cations by extraction and fractionation of the chloroplast rRNA at temperatures between 0° and 5°C. On increasing the temperature of rRNA prepared at a low temperature, fragmentation to characteristic products is observed, the T_m ('melting' temperature) of which is markedly affected by ionic strength and the presence of bivalent cations.

Methods

Broad-bean (*Vicia faba* var. Midget) plants were grown under normal greenhouse conditions, and leaves from 14-day-old plants were used for preparation of chloroplasts and nucleic acids as previously described (Leaver & Ingle, 1971), except that Mg^{2+} was omitted from the buffer used for isolation and the temperature of the preparations was kept below 4°C.

The chloroplast RNA preparations were washed three times by resuspension in cold 80% (v/v) ethanol followed by low-speed centrifugation. They were then either used directly or fractionated further by centrifugation at $49500 g_{av.}$ for 18h at 2°C, in a Spinco SW25.1 rotor, on linear 5–20% (w/v) sucrose gradients in 0.5% sodium dodecyl sulphate–0.15M-lithium acetate, pH6.0. The heavy (1.1×10^6-mol.wt.) and light (0.56×10^6-mol.wt.) rRNA components were recovered from the gradients by precipitation with 2.5vol. of cold absolute ethanol and collected by centrifugation. The purified rRNA components were washed three times by resuspension and centrifugation in cold 80% ethanol. The nucleic acid preparations were dissolved in electrophoresis buffer (E buffer: 30mM-NaH_2PO_4, 36mM-Tris and 1mM-EDTA, pH8.0) at a concentration of 1.0mg/ml and below 4°C.

RNA preparations were fractionated by polyacrylamide-gel electrophoresis in E buffer as previously described (Leaver & Ingle, 1971), except that the sodium dodecyl sulphate concentration in the electrophoresis buffers was in all cases 0.05% and electrophoresis conditions were as described in the figure legends.

The molecular weights of the RNA components were derived from co-electrophoresis with *Escherichia coli* rRNA species (1.1×10^6 and 0.56×10^6 mol.wt.) as standards.

Results and discussion

A densitometer scan of a gel containing chloroplast rRNA extracted and subjected to electrophoresis at a temperature not exceeding 5°C (Fig. 1*a*) shows that the ratio of heavy (1.1×10^6-mol.wt.) to light (0.56×10^6-mol.wt.) rRNA components (1.86:1) is close to the value of 1.98:1 that is expected if the two rRNA species are present in equimolar amounts in chloroplast ribosomes. Gel electrophoresis under similar conditions of gradient-purified 1.1×10^6-mol.wt. rRNA and 0.56×10^6-mol.wt. rRNA (Figs. 1*b* and 1*c*) indicates that they were intact and virtually

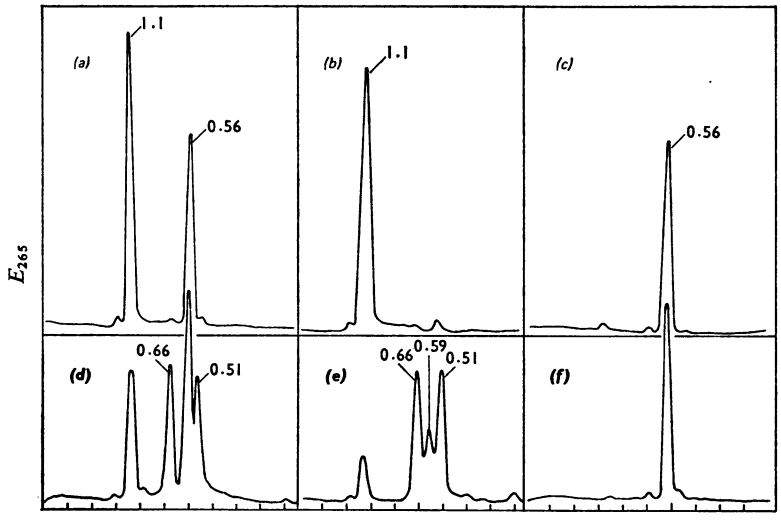

Fig. 1. *Effect of temperature pretreatment on the stability of chloroplast rRNA species*

Samples of total broad-bean chloroplast rRNA (*a* and *d*), 1.1×10^6-mol.wt. rRNA (*b* and *e*) and 0.56×10^6-mol.wt. rRNA (*c* and *f*) were exposed for 5 min to temperatures of either 5°C (*a–c*) or 40°C (*d–f*) followed by rapid cooling and electrophoresis in E buffer on 2.4% polyacrylamide gels for 4 h at 50 V (3 mA/9 cm gel) and a temperature of 5°C.

homogeneous. If rRNA samples similar to those analysed as described in Figs. 1(*a*)–1(*c*) are heated at 40°C for 5 min, gel profiles as illustrated in Figs. 1(*d*) (total chloroplast rRNA), 1(*e*) (1.1×10^6-mol.wt. rRNA) and 1(*f*) (0.56×10^6-mol.wt. rRNA) are obtained. In the total chloroplast rRNA preparation the ratio of the 1.1×10^6-mol.wt. rRNA to 0.56×10^6-mol.wt. rRNA is 0.62:1, indicating major breakdown of the 1.1×10^6-mol.wt. rRNA component. Electrophoresis of the heat-treated 1.1×10^6-mol.wt. rRNA (Fig. 1*e*) indicated the presence of the three major cleavage products of mol.wt. 0.66×10^6, 0.59×10^6 and 0.51×10^6. The 0.56×10^6-mol.wt. rRNA (Fig. 1*f*) was completely stable to the 40°C temperature treatment.

Similar results are obtained if the cold-extracted RNA is exposed to 6M-urea at low temperature and subsequently analysed by electrophoresis.

These observations show that most of the 1.1×10^6-mol.wt. rRNA molecules, as prepared by standard methods of extraction, are nicked at several specific points along the polynucleotide chain, the fragments being held together at low temperature by secondary structure. Conditions, e.g. heating or treatment with urea, that dissociate hydrogen bonds and lead to a loss of secondary structure cause dissociation of the molecule.

To obtain some idea of the degree of secondary structure (hydrogen-bonding), suitable samples of the purified 1.1×10^6-mol.wt. rRNA were exposed in E buffer for a 5 min period to increasing temperatures over the range 5–50°C, and then rapidly cooled in ice. The samples were subjected to electrophoresis in the cold and the percentages of the 1.1×10^6-mol.wt. rRNA component remaining were calculated. It can be seen from Fig. 2 that at 5°C the 1.1×10^6-mol.wt. rRNA component is essentially 100% intact, but that as the temperature of pretreatment is increased more of the molecules are dissociated. The temperature required to produce 50% dissociation in E buffer (T_m) is 24°C, indicating relatively short regions of hydrogen-bonding holding the nicked molecules together. The persistence of approximately 10% intact 1.1×10^6-mol.wt. rRNA, taken together with previous evidence that newly synthesized 1.1×10^6-mol.wt. rRNA is stable under similar conditions (Ingle, 1968), suggests that there are few intact 1.1×10^6-mol.wt. rRNA molecules in the mature chloroplast and that nicks are introduced at an early stage after ribosome synthesis.

Inclusion of 10mM-Mg^{2+} in the E buffer during temperature pretreatment of the rRNA (Fig. 2) increased the temperature of dissociation of the 1.1×10^6-mol.wt. component so that the T_m is at

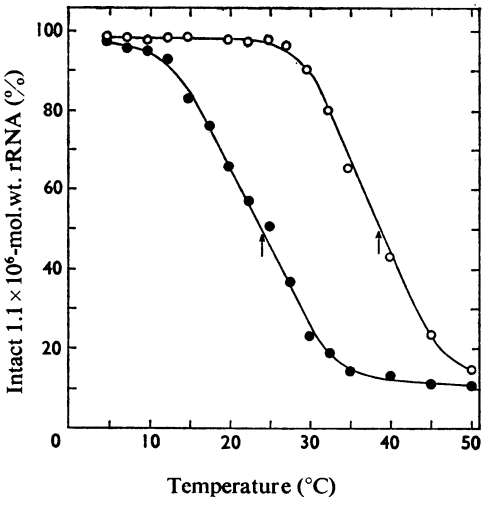

Fig. 2. *Effect of increasing temperature on the stability of chloroplast* 1.1×10^6*-mol.wt. rRNA*

Chloroplast 1.1×10^6-mol.wt. rRNA was dissolved in E buffer (●) or in E buffer containing 10mM-Mg^{2+} (○), and portions were exposed for 5min to increasing temperatures in the range 5–50°C, followed by rapid cooling and electrophoresis at 5°C (as described in Fig. 1). The percentages of intact 1.1×10^6-mol.wt. rRNA remaining after the different temperature treatments were calculated from the gel profiles and are plotted as a function of temperature of pretreatment. The temperatures at which 50% intact 1.1×10^6-mol.wt. rRNA remains are indicated by the arrows.

38°C. The increased thermal stability conferred on the rRNA by bivalent cations explains why we were able to obtain apparently intact 1.1×10^6-mol.wt. rRNA even at room temperatures (Leaver & Ingle, 1971).

A similar stabilizing effect was obtained by Rawson *et al.* (1971), who has presented evidence that the cytoplasmic 25S (1.3×10^6-mol.wt.) rRNA from *Euglena* is cleaved at selected loci *in situ* and that the inclusion of Na^+ in the isolation buffer stabilizes the 25S rRNA. A short incubation at 37–45°C was, however, found to cause extensive degradation, independent of the ionic conditions used during the isolation procedure.

The stabilizing effect of bivalent cations is perhaps partially explained by previous observations made by other workers. Fuwa *et al.* (1960) demonstrated that ions of metals of the first transition period, including Mg^{2+} and Ni^{2+}, maintain the secondary helical structure of RNA molecules through the formation of intramolecular bonds, this stabilization even occurring when hydrogen bonds are being disrupted by, for example, an increase in temperature. By analogy with the well-documented effect of tertiary folding in proteins, the metal ion is envisaged as serving as a cross-link between nucleotide residues on adjacent turns of the helix, thus fixing or stabilizing the hydrogen-bonded secondary structure enclosed within the tertiary loop. Spirin (1960) has suggested that these internal linkages are of the phosphate–metal ion–phosphate or purine–metal ion–phosphate bonding type.

Goldberg (1966), from his own work and quoting observations made by Boedtker (1966) and Felsenfeld (1962), suggests that Mg^{2+} is several orders of magnitude more effective than univalent ions in assisting the formation of specific secondary structure in nucleic acids, and that stabilization of ordered polynucleotide structures requires that much of the phosphate backbone charge be neutralized or screened.

The observations reported in the present communication support the conclusion that, under normal conditions in the intact chloroplast 70S ribosome, the 1.1×10^6-mol.wt. rRNA molecule contains considerable secondary structure in the form of helical regions or base-paired loops stabilized by hydrogen bonds. Any loops exposed at the surface of the large ribosomal subunit will be liable to endonuclease action *in situ* or during the isolation of RNA, resulting in the introduction of nicks or short deletions into the polynucleotide chain. If the base-paired region of the loop is large enough, the isolated RNA molecule in solution will remain intact, provided that the secondary structure is preserved by ionic and temperature conditions (high salt concentrations and low temperatures) that encourage hydrogen-bonding and/or the stabilization of the molecule by bivalent cations.

Under conditions that progressively abolish hydrogen-bonding and cause unfolding of the RNA molecule, e.g. increase in the temperature, lowering of the salt concentration or inclusion of urea in the solution, the secondary structure of the RNA molecule is lost and the molecule dissociates into several shorter polynucleotide chains. Once the shorter polynucleotide constituents of the 1.1×10^6-mol.wt. rRNA have dissociated from one another they cannot, apparently, be induced to reassociate (C. J. Leaver, unpublished work).

The difference in stability of the two rRNA components is probably best explained on the grounds of selective nuclease action occurring either *in situ* or during the deproteinization step. The 0.56×10^6-mol.wt. rRNA obviously differs, in base sequence, configuration or nucleic acid–protein interaction in the subunit, from the 1.1×10^6-mol.wt. rRNA.

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