Mitochondrial nucleoproteins in rye leaves

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Abstract:

Some properties of mitochondrial nucleoproteins sedimentable at 100 000 g, corresponding to the hyaloplasmic ribosomes, were compared with those found for two other subfractions isolated from mitochondria of primary rye (*Secale cereale*) leaves, the pellet sedimentable at 20 000 g and the supernatant remaining after 100 000 g.

INTRODUCTION

The presence of nucleic acids within mitochondrion has been firmly established (cf. Gołaszewski et al., 1969). High- and low-molecular-weight RNA species in the organella were repeatedly found in various organisms. The role of mitochondrial DNA in extrachromosomal inheritance has been recognized (Nass, 1969) but numerous conflicting data on synthesis of mitochondrial proteins do not allow to explain the role of mitochondrial RNA's. According to Work et al. (1968) only about 30% of mitochondrial proteins are supplied by mitochondrial protein synthesizing system. Kadenbach (1967) and Beattie (1968) have made clear that some of the mitochondrial proteins are being formed in cytoplasm on hyaloplasmic ribosomes.

The existence of mitochondrial ribosome-like particles was stated by Rendi (1959) in rat liver cells. Ribosomes in mitochondria of yeasts (Wintersberger, 1967) and of fungus *Neurospora crassa* (Küntzel, 1969; Küntzel and Noll, 1969) were found to be different from those in respective cytoplasm. Ribosomal RNA from mitochondria in those species was investigated, too. On the contrary, mitochondria originating from higher plants were not widely investigated in this respect.

In this paper we present some data on mitochondrial nucleoproteins (corresponding to hyaloplasmic ribosomes) from mitochondria of primary rye (*Secale cereale*) leaves. Some properties of RNA isolated from those

nucleoproteins are compared to properties of RNA of the other mitochondrial subfractions and of hyaloplasmic ribosomes. The distribution of DNA within mitochondrion was followed, as well.

MATERIAL AND METHODS

Plants. Rye (*Secale cereale*) var. Dańkowskie was cultivated under normal light regime, as described previously (Ombach et al., 1966); 14-day-old seedlings were used. Primary leaves freezed on dry-ice were cut into 1 cm fragments and homogenized with a grinding medium (in proportion 100 ml/200 g of leaf tissue, wet weight) containing 0,25 M saccharose, 0,01 M magnesium acetate and 0,01 M Tris-HCl buffer, pH 7,3. in the Turmix homogenizer at maximum speed for 1 min. The homogenate was filtered through four layers of cheese-cloth and subjected to differential centrifugation.

Isolation of mitochondria and hyaloplasmic ribosomes. After preliminary centrifuging at 1000 g for 14 min to sediment the cell debris, nuclei and chloroplasts, the resultant supernatant was centrifuged at 12 000 g for 15 min to sediment crude mitochondrial fraction. It was resuspended in 15 ml of grinding medium with a teflon pestle and, after centrifuging at 1000 g for 14 min to remove the plastidial impurities, mitochondria were spun down at 12 000 g for 15 min. All steps of the procedure were carried out in the cold (4°C).

From the post-mitochondrial supernatant hyaloplasmic ribosomes were spun down at 105 000 g (calculated at maximal radius basis) for 2 hr. Spinco Model L-2 ultracentrifuge, Rotor No 20 was used. All details concerning the procedure were described previously (Gołaszewski et al. 1967).

Subfractionation of mitochondria. The mitochondrial pellet suspended in 5 ml of the grinding medium (ca. 7 mg of protein per ml) was subjected to sonic oscillations with use of a MSE, 100 W, Ultrasonic Disintegrator (20 kcyc./sec., three 1 min periods interspaced by 30 sec. intervals) at 2°. The homogenate was made up to 20 ml with the above medium and it was subjected to differential centrifugation according to scheme generally used for standard differential centrifugation technique for cell fractionation. Three mitochondrial subfractions were obtained; 1. 20 000 g pellet (for 20 min); 2. 20 000 — 100 000 g pellet (for 2 hr) and 3. the supernatant remaining after 100 000 g (the 100 000 g supernatant). To the latter subfraction, solid sodium chloride up to 0,1 M of the final concentration and 3 vol. of cold 96% ethanol were added. After 24 hr nucleoproteins precipitated were sedimented. For the sake of comparison, in some experiments, mitochondrial "ribosomal" subfraction was obtained under conditions described by K ü n t z e l (1969) for mitochondrial ribosomes in *Neurospora crassa*. Mitochondria isolated from primary rye leaves as de-

scribed above were suspended in 0,01 M Tris-HCl buffer, pH 7,5 containing 0,6 M NH_4Cl and, 0,014 M MgCl_2 and Triton X-100 (Rohm and Haas, Philadelphia, USA) up to 2% of final concentration was added. After lysis, differential centrifugation of mitochondrial homogenates was carried out. After preliminary centrifuging at 26000 g for 20 min. the resultant supernatant was centrifuged at 100 000 g for 2 hr to sediment the pellet of nucleoproteins corresponding to mitochondrial ribosomes. The pellet obtained was suspended in 0.25 M saccharose buffered with 0.01 M Tris-HCl buffer, pH 7.5 containing 0.01 M MgCl_2 and 0.1 M H_4Cl . From the pellet RNA was isolated with phenolic method (Click and Hackett, 1966) and it was put into the MAK column (*vide infra*).

Isolation of RNA. Mitochondrial RNA from the 100 000 g pellet and RNA from hyaloplasmic ribosomes was isolated by phenolic method described by Click and Hackett (1966), using 0.1 M buffer Tris-HCl, pH 7.3 (Rytel et al., 1969 c).

Fractionation of RNA on methylated albumin-kieselguhr (MAK) column. In these experiments methylated albumin covered with infusorial earth was prepared according to Mandell and Harshy (1960) and placed in a glass column fitted with sintered glass filter disc (Schott G-3). The column was connected to a water pump and equilibrated with 0.1 M NaCl containing 0.05 M phosphate buffer, pH 6.7 according to Sueoka and Cheng (1962). The size of the column was $3 \times 1,2$ cm. The solutions of RNA isolated from the 100 000 g pellet were applied into the column in 0.05 M phosphate buffer, pH 6.7. Substances nonabsorbable on methylated albumin were washed off with 0.1 M NaCl in 0.05 M phosphate buffer, pH 6.7. RNA was eluted from the column with sodium chloride solutions (0.2—1.3 M) in the same buffer. Fractions eluted in the range of NaCl concentrations 0.2—0.7 M (low-molecular-weight RNA) and 0.8—1.3 M (high-molecular-weight RNA) were separately pooled, dialysed against 0.001 M phosphate buffer, pH 6.7 and lyophilized to a small volume.

Absorbance melting profiles of RNA. Melting properties of RNA in solution were followed by heating 1 ml samples in 1 ml cuvettes in a Unicam SP-500 spectrophotometer equipped with a thermoregulated cuvette holder. The temperature was controlled with water-glycerol mixture from a Hoeppler ultrathermostat. As the medium the mixture consisting of 0,15 M sodium chloride and 0,015 M sodium citrate (SSC medium), pH 7,0 (Doty et al. 1959) was used.

Analytical methods

RNA isolated by phenol method (Click and Hackett, 1966) was estimated spectrophotometrically, absorbancy at 260 m μ was read, an

extinction coefficient of $24,0 \text{ mg}^{-1} \cdot \text{ml}^{-1} \cdot \text{cm}^{-1}$ was applied (Nathans and Lipmann, 1961).

In experiments when DNA/RNA ratios were estimated RNA was extracted with Ogur and Rosen's method (1950) with cold (4°) 1 N perchloric acid for 6 hr (Gołaszewski et al., 1967) and calculations were made according to Nieman and Poulsen (1967). DNA was extracted from the residue (after RNA extraction) with 0,5 N perchloric acid at 70° for 15 min (Nieman and Poulsen, 1967) and it was estimated with diphenylamine reaction (Dische, 1955). Calf-thymus DNA (Type I, Sigma Chemical Co., USA) was used as the standard.

RESULTS

As is it can be seen from the Table 1 the 100 000 g pellet obtainable from the mitochondria homogenates by use of differential centrifugation technique differs from two other mitochondrial subfractions studied. Its DNA/RNA ratio was found to be the lowest one in comparison to those found for the other mitochondrial subfractions. The highest DNA/RNA ratio was found for nucleoproteins present in the 100 000 g supernatant. On comparing DNA contents in various mitochondrial subfractions in refer to 1 mg of the ether powder prepared from them it can be seen (Table 1) that the 100 000 g pellet contain the lowest DNA amounts. The highest one was found for the 100 000 g supernatant nucleoproteins.

RNA isolated from mitochondrial 100 000 g pellet comprises mainly high-molecular-weight molecules, elutable from MAK column with sodium chloride solutions in the range of concentrations 0,8—1,3 M (Sueoka and Cheng, 1962). As it can be seen from the Fig. 1 high

Table 1

Distribution of DNA within mitochondrion of primary rye leaves

Mitochondrial subfraction	DNA/RNA ratio *	DNA/mg of ether powder ** ($\mu\text{g } \mu\text{g}$)
1. 20 000 g pellet	0.06	2.6
2. 100 000 g pellet	0.04	1.9
3. 100 000 g supernatant ***	0.1—0.2	2.8
Unfractionated mitochondria	0.07	2.3

* RNA was hydrolysed acc. to Ogur and Rosen (1950) with cold 1 N perchloric acid, as described previously (Gołaszewski et al. 1967). Determinations of RNA acc. to Nieman and Poulsen (1967). The ether powders were prepared acc. to Nieman and Poulsen (1963). From the residue after extraction of RNA, DNA was extracted with 0,5 N perchloric acid at 70° for 15 min. acc. Nieman and Poulsen (1963). Diphenylamine reaction was carried out acc. to Dische (1955) to determine DNA contents. Calf thymus DNA was used as a standard.

** The ether powders prepared acc. to Nieman and Poulsen (1963). DNA determinations, as for *)

*** Before determinations, solid sodium chloride up to 0,1 M of final concentration and 3 vol. cold 96% ethanol were added to precipitate the nucleoproteins and the ether powders were prepared from the pellet obtained. The data in the Table 1 represent the average values of six experiments (separate preparations in each case).

Fig. 1. Diagram of MAK column fractionation of RNA isolated from mitochondrial 100 000 g pellet

Experimental conditions, see Material and Methods. Mitochondrial 100 000 g pellet was obtained after sonic disintegration of mitochondria. 0.5 mg of RNA ($E_{260}^{1\text{ cm}} = 12$) was put into the column filled with methylated albumin covered with infusorial earth (MAK) and equilibrated with 0.1 M sodium chloride solution in 0.05 M phosphate buffer, pH 6.7. Size of the column: 1.2×3 cm; The column was washed successively with 0.2 M, 0.7 M and 1.3 M sodium chloride solutions in the same buffer; 1 ml fractions were collected, 15 ml of eluent for each NaCl concentration were applied. $E_{260}^{1\text{ cm}}$ was read with an Unicam SP-500 spectrophotometer. The total $E_{260}^{1\text{ cm}}$ for two molecular RNA forms is presented. The RNA recovery — ca. 80%.

molecular-weight RNA constitutes in this mitochondrial subfraction ca 75% of RNA elutable from the column. Similar elution patterns for RNA isolable from the 100 000 g pellet were obtained no matter what method of disintegration of mitochondria was used, K ü n t z e l's method (1969) with use of Triton X-100 (to solubilize mitochondrial membranes) or the sonic disintegration of the organella, as described above (cf. R y t e l et al., 1970).

The temperature profiles for RNA isolated from the 100 000 g mitochondrial pellet and from hyaloplasmic ribosomes from the same source show some differences in stability of their molecules. So called melting points (T_m) found for the two RNA's are respectively 52° and 47°. The "melting" profiles for RNA of the 100 000 g mitochondrial pellet and hyaloplasmic ribosomes are presented in the Fig. 2. The per cent increase in absorbancy measured at 260 mμ as a function of temperature (hyper-

Fig. 2. Absorbance melting profiles of RNA from mitochondrial and hyaloplasmic ribosomes of rye leaves

1 — RNA of mitochondrial nucleoproteins; 2 — RNA of hyaloplasmic ribosomes
Absorbance melting profiles were run in an Unicam SP-500 spectrophotometer, equipped with 3 cuvette brass block compartment. The temperature was controlled with water-glycerol mixture from a Hoeppler ultrathermostat. Quartz cuvettes were equipped with tightly fit plastic corks. SSC medium (Doty et al. 1959) was used. The changes in E_{260} were checked every two degrees. The dashed lines indicate T_m for RNA's isolated by phenolic method (Click and Hackett, 1966) from mitochondrial 100 000 g pellet (52°) and hyaloplasmic ribosomes (47°). Similar results were obtained both for total RNA from mitochondrial ribosomes and ribosomal RNA eluted from MAK column. After cooling decrease in E_{260} readings to initial values was observed.

Table 2

Comparison of the temperature profiles of RNA's of mitochondrial and hyaloplasmic ribosomes

Observation	Source of rRNA	
	Ribosomes	
	Mitochondrial	Hyaloplasmic
"Melting" point (T_m)	52°	47°
Hyperchromasity effect (ΔE_{260})	21%	23%
Initial-final temperatures ΔE_{260}	35° — 70°	35° — 60°

Experimental conditions, see legend to the Fig. 2.

chromasity) was found to be almost the same for both RNA's, and it was about 20%. Although the initial temperature at which the absorbancy increase started was the same in both cases (35°), the final temperature of absorbancy increase was higher for mitochondrial 100 000 g RNA (see Table 2). Similar results were obtained when either total RNA isolable from the 100 000 g pellet or solely highly polymerized RNA eluted from MAK column were checked. In both cases the decrease in absorbancy at 260 m μ to initial values was observed after cooling.

DISCUSSION

RNA isolable from the 100 000 g pellet of rye leaf mitochondria comprises two main molecular forms: low- and high-molecular-weight RNA's. They can be separated on the MAK columns. About 80% of RNA eluted from the column appeared to represent high-molecular-weight RNA. Thus, mitochondrial 100 000 g pellet contains RNA whose MAK column separation profile is quite different from that obtainable for RNA isolated from the 20 000 g pellet subfraction of rye leaf mitochondria (Rytel et al., 1970) and for RNA extracted from unfractionated mitochondria (Rytel et al., 1969 c). In the latter cases about 70% of RNA could be eluted from the MAK column with 0.2—0.7 sodium chloride solution in phosphate buffer as low-molecular-weight RNA's. As far as RNA of the 100 000 g supernatant is concerned, proportions of both forms varied from one experiment to another, but high molecular forms never exceeded 60% of the total RNA extracted from that mitochondrial subfraction. The discrepancies noted, most probably, were due to high ribonucleolytic activity found in that mitochondrial subfraction (Rytel et al., 1969 a).

The studies on the "melting" profiles of RNA's as a measure of relative stability of their helices show that RNA isolated from the 100 000 g pellet of rye leaf mitochondria is a little more stable than RNA isolated from hyaloplasmic ribosomes originating from rye leaf cells. "Melting" temperature (T_m) for RNA's from both sources appeared to be 52° and 47°, respectively. $T_m = 52^\circ$ was stated for RNA both when total RNA isolable from the 100 000 g pellet and solely high-molecular-weight RNA of that mitochondrial subfraction (after MAK column separation) were checked. On the other hand RNA from the 100 000 g mitochondrial supernatant did not show any hyperchromasity. Our previous results (Rytel et al., 1969 c) pointed to some similarities of mitochondrial RNA and RNA of hyaloplasmic ribosomes in nucleotide composition. The results presented in this paper show, however, that RNA of mitochondrial nucleoproteins corresponding to hyaloplasmic ribosomes in rye leaves constitutes separate RNA pool in the cell.

Mitochondrial nucleoproteins sedimentable at 100 000 g contain lower amounts of DNA in comparison to those found in the 20 000 g pellet and

in the 100 000 g supernatant subfraction. The latter one was found to contain relatively high amount of DNA in refer to RNA what is visualized by high DNA/RNA ratio ranging from 0,1 to 0,2. For mitochondrial 100 000 g pellet DNA/RNA ratio was found to be the lowest one among those found either for the other mitochondrial subfractions or unfractionated mitochondria. The same is true when DNA amounts found in mitochondrial subfractions are calculated on the dry weight basis of the ether powders prepared from them. These results point to that mitochondrial 100 000 g pellet constitutes a subfraction which differs both from the 20 000 g pellet and the 100 000 g supernatant. From our previous results (Rytel et al., 1969 a) it is known that the 100 000 g pellet subfraction shows relatively high (0,3) RNA/Protein ratio in opposite to the other mitochondrial subfractions. Thus, the 100 000 g pellet appeared to be „enriched” in RNA. On the other hand, it differs significantly in this respect from hyaloplasmic ribosomes originating from the same plant species (Gołaszewski et al., 1967). Thus, the question arises what is the role of mitochondrial nucleoprotein subfraction that differs from both the other mitochondrial subfractions and hyaloplasmic ribosomes. In our previous papers (Szarkowski et al., 1969; Rytel et al., 1969 b; Rytel et al., 1970) we have presented several lines of evidence that *in vitro* mitochondrial 100 000 g pellet shows relatively high RNA turnover while it only slightly incorporates labelled amino acids into mitochondrial proteins. When separated mitochondrial subfractions were incubated with labelled [^{14}C]-uracil that was only the 100 000 g supernatant that exhibited high RNA turnover. In this paper we have stated that this mitochondrial subfraction is characterised by high DNA/RNA ratio. So, it seems reasonable to assume that RNA synthesized in the 100 000 g supernatant subfraction is then transported to and accumulated in the 100 000 g pellet. If this is so, we would deal with some sort of compartmentation of RNA metabolism within the organella.

We have found previously (Rytel et al., 1969 a) that mitochondrial 100 000 g pellet compares only ca. 10% of the total amount of RNA found in rye leaf mitochondria. It is commonly known (cf. Gołaszewski and Szarkowski, 1966) that hyaloplasmic ribosomes, no matter where they originate from, contain the bulk of cytoplasmic RNA. This is one more feature that points to the differences existing between the 100 000 g pellet and hyaloplasmic ribosomes, at least in higher plants. In this connection we express our opinion that the term “mitochondrial ribosomes”, in case of higher plants, is not correct.

CONCLUSIONS

The comparison of mitochondrial nucleoproteins sedimentable at 100 000 g, corresponding to hyaloplasmic ribosomes, with the pellet sedi-

mentable at 20 000 g and the supernatant remaining after 100 000 g has shown:

1. The 100 000 g pellet shows the lowest DNA/RNA ratio (Table 1). Absorbance melting profile of RNA isolated from the 100 000 g mitochondrial pellet indicates that "melting point" for this RNA is 5 degrees higher than that found for RNA isolated from hyaloplasmic ribosomes (Fig. 2).

2. In opposite to the other mitochondrial subfractions, the 100 000 g pellet contains RNA consisting of highly polymerised forms, mainly (Fig. 1).

3. In this paper the role of mitochondrial nucleoproteins sedimentable at 100 000 g is discussed and the term "mitochondrial ribosomes" is criticized.

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Nukleoproteidy mitochondrialne w liściach żyta

Streszczenie

Niektóre własności osadu nukleoproteidów mitochondrialnych opadających przy 100 000 g, odpowiadających rybosomom hialoplazmatycznym, porównano z własnościami dwóch pozostałych podfrakcji izolowanych z mitochondriów pierwszych liści żyta (*Secale cereale*): osadu opadającego przy 20 000 g i supernatantu pozostającego po 100 000 g. Osad 100 000 g charakteryzuje się najniższym stosunkiem DNA/RNA (Tab. 1). Profil termiczny RNA izolowanego z osadu 100 000 g mitochondriów wskazuje, że temperatura „topnienia” tego RNA (52°) jest wyższa o 5° niż analogiczna wartość stwierdzana dla RNA izolowanego z rybosomów hialoplazmatycznych (47°) (Fig. 2). W przeciwieństwie do pozostałych podfrakcji mitochondrialnych, osad 100 000 g zawiera głównie wysokopolimeryzowany RNA (Fig. 1). W pracy dyskutowana jest rola nukleoproteidów mitochondrialnych opadających przy 100 000 g, przy czym stwierdza się niepoprawność terminu „rybosomy mitochondrialne”.