A SENSITIVE IN VITRO PROTEIN SYNTHESIZING SYSTEM FROM EHRLICH ASCITES MITOCHONDRIA

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Summary: The S-25 fraction prepared from digitonin washed mitochondria is highly active in poly(U) directed phenyalanine incorporation when supplemented with t-RNA. Ribosomes prepared from the S-25 fraction contain 58S monomeric ribosomes and 40S and 29S subunits. Further, these ribosomes contain 21S and 13S rRNA. No detectable cytoplasmic specific ribosomal particles and also rRNA was detected in the mitochondrial S-25 preparation. Ribosomes from mitochondrial S-25 have specific requirement for mitochondrial specific supernatant factors for complete activity.

Introduction

It is now well established that mitochondria from diverse cell types contain autonomous protein synthesizing systems quite distinct from the cytoplasmic counterparts (1,2,3). While the detailed characterization of molecular processes involved in the cytoplasmic specific protein synthesis is gaining momentum (4), studies on mitochondrial specific protein synthesis has been fragmentary, partly because of the lack of an active in vitro system from these organelles. Problems involved with the successful isolation of active in vitro systems from mitochondria are manifold. First of all, the problem of cross-contamination with cytoplasmic fragments, especially in the case of mammalian cells, is a major concern. Consequently, most techniques to purify mitochondria use repeated washings with sucrose-EDTA which in turn contributes to the reduced activity of mitochondrial lysates. In this paper we describe a rapid two step digitonin washing technique to prepare mitochondria essentially free of cytoplasmic specific ribosomes. Lysing these mitochondria under controlled conditions allows the preparation of S-25 fraction highly active in phenylalanine incorporation directed by poly(U). Similar to its cytoplasmic counterpart (5) the activity of the mitochondrial system is dependent on the addition of tRNA to the reaction mixture.

Materials and Methods

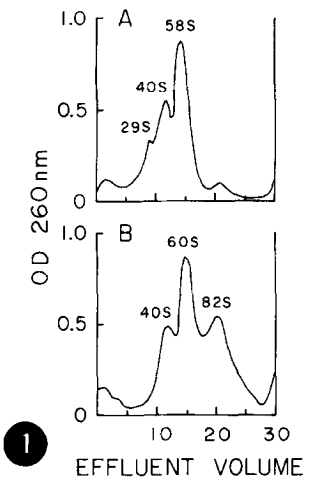
Preparation of Mitochondria: Mitochondria were prepared from Ehrlich ascites hypertetraploid cells grown for seven days in the peritoneal cavity of Swiss colony mice essentially as described before (6). In each experiment, cells from 250-300 tumor-bearing mice (10^{12}-10^{13} cells) were used for mitochondrial preparation. Cells were homogenized in a buffer (HB) containing 300 mM sucrose-10 mM Tris-HCl (pH 7.5)-120 mM KCl-2 mM EDTA. Nuclei were removed by two successive centrifugations at 1100 x g for 8 min. Mitochondria were then pelleted at 7,500 x g for 15 min. Crude mitochondrial pellet was washed two times with digitonin (Sigma), first with a 0.5% solution in HB and then with 1% digitonin in HB. Mitochondrial pellet was finally washed once with 30 mM Tris-HCl (pH 7.5)-120 mM KCl-15 mM Mg $(CH_3COO)_2$-7 mM 2-Mercapto ethanol (BRS) containing 0.25 M sucrose and used for preparing S-25.

Preparation of S-25 Purified mitochondria were suspended in two packed volumes of BRS and homogenized with 10 strokes of a tighly fitted dounce homogenizer. DNase (RNase-free, Worthington) was added at a final concentration of 2 μg/ml. The homogenate was made to 0.5% final concentration with nonidet NP40 (Shell Oil Co.) and homogenized with 5 more strokes. After 5 min at 0-4°C, the homogenate was centrifuged at 25,000 x g for 15 min and the upper 3/4 of the supernatant was aspirated out. The supernatant was then adjusted to final concentrations of 0.5 mM ATP, 0.1 mM GTP 0.3 mM CTP, 3 mM Phosphoenol pyruvate (PEP), 40 μM each of 20 amino acids, 1.6 μg/ml Pyruvate Kinase and incubated at 35°C for 20 min. Pre-incubated extract was clarified at 10,000 x g for 10 min and passed through a Sephadex G-25 column (0.8 cm x 25 cm) equilibrated with BRS. At this stage, the extract (S-25) had 36-45 units/ml of O D at 260 nm.

Preparation of Ribosomes and Factors: Ribosomes and ribosome-free factors were prepared by centrifuging S-25 over 1.0 M sucrose as described by Aviv et al (5). Factors from E.coli and Ehrlich ascites cytoplasm were prepared by centrifuging the respective S-30 fractions over 1.0 M sucrose as described above.

Results

In order to verify the purity of the mitochondrial extracts, the ribonucleo-



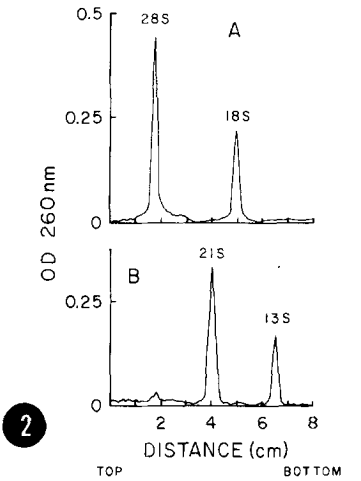


Fig. 1 Density gradient analysis of Ehrlich ascites Mitochondrial and cytoplasmic ribosomes. About 5-10 A_{260} units of ribosomes prepared from mitochondrial S-25 (A) and cytoplasmic S-30 (B) were layered on 29.5 ml of 10-36% sucrose gradients containing 20 mM Tris-HCl (pH 7.4)-80 mM KCl-2 mM $MgCl_2$ and centrifuged at 76,000 x g for 3 1/2 hrs. (4-6°C). E. coli ribosomes were used as markers.

Fig. 2 Electrophoresis of phenol-SDS extracted ribosomes from cytoplasmic (A) and mitochondrial S-25 (B). About 15-20 ug RNA was layered on 3.0% polyacrylamide gels and electrophoresed as described before (5). Gels were scanned at 260 nm using a Gilford spectrophotometer.

protein particles pelleted by centrifuging the S-25 over a cushion of 1.0 M sucrose, were analyzed on 10-35% linear sucrose gradients. As shown in Fig. 1, ribosomes from mitochondrial extracts resolve into 58S, 40S and 29S peaks indicating that 40S and 29S may be the subunits of 58S mono-ribosomes. Though there is no detectable optical density at the 80S region, presence of some cytoplasmic specific ribosomal subunits sedimenting at 60S and 40S cannot be excluded. Presence of cytoplasmic ribosomal subunits, in the mitochondrial extracts can, however, be easily verified by electrophoretic analysis of rRNA's, since mitochondrial rRNA's of diverse origin are known to migrate much faster than the cytoplasmic rRNA's (7). Electrophoretogram presented in Fig. 2 shows that rRNA prepared from the mitochondrial extracts migrate

TABLE 1: ACTIVITY OF MITOCHONDRIAL S-25

Details	^3H CPM incorporated x 10^{-3}		
	Expt I	Expt II	Expt III
S-25	1.9	2.0	2.1
+ Poly(U)	22.4	21.3	21.8
+ tRNA	2.6	2.1	2.8
+ Poly (U) and tRNA	215.6	209.3	206.9

Incorporation assays were run at 35°C for 30 min in 0.05 ml final volumes. Reaction was run in BRS containing 0.5 mM ATP, 0.1 mM GTP 0.3 mM CTP, 3 mM PEP, 1.6 µg pyruvate kinase, 0.5 µci ^3H labeled phenyl alanine (New England Nuclear, 25 ci/mmole) and 225-250 µg S-25 protein. Poly(U) (Miles laboratory) and tRNA from yeast (Sigma) when added were at the concentrations of 0.5, and 1.0 OD 260 nm units respectively. Other details were as described in Aviv et al (5).

as 21S and 13S peaks. No significant optical density is detected in the regions of 28S and 18S indicating the purity of mitochondrial S-25.

As shown in Table 1, the S-25 extract from Ehrlich ascites mitochondria is highly active in incorporating ^3H phenylalanine into hot-CCl_3COOH insoluble fraction. Tube without added poly(U) has insignificant counts indicating negligible or no endogenous mRNA in the S-25. Further, complete incorporation activity is also dependent upon the addition of tRNA.

As shown in Table 2, mitochondrial in vitro system requires ATP, GTP and phosphate generating systems. In addition, the system is sensitive to streptomycin, chloramphenicol and Puromycin, as expected (1,2,3). The mitochondrial protein synthesis, however, is unaffected by cycloheximide and Emetine which are known to be the specific inhibitors of 80S type eukaryotic ribosomal systems (8).

Finally, in order to verify any specificity of factor requirement, experiments were carried out using mitochondrial ribosomes and supernatant factors from mitochondria, cytoplasm and E.coli. As shown in Table 3, factor from homologous source, i.e., mitochondria, yields maximum activity, comparable to that obtained with mitochondrial S-25. On the other hand, mitochondrial ribosomes show about

TABLE 2: EFFECTS OF OMISSIONS OF TRIPHOSPHATES AND ADDITIONS OF ANTIBIOTICS ON IN VITRO PROTEIN SYNTHESIS

Additions or omissions	CPM x10^{-3}		
	Expt I	Expt II	Expt III
None	245.8	224.1	227.1
- ATP	21.4	28.0	25.2
- GTP	45.6	39.7	48.7
- PEP and Kinase	98.2	92.7	90.8
+ Puromycin	49.8	52.6	53.0
+ Emetine	219.4	208.5	206.8
+ Cycloheximide	201.2	205.3	202.8
+ Streptomycin	162.9	163.1	165.8
+ Chloramphenicol	81.6	77.5	79.6

Details of in vitro protein synthesis assays were as in Table 1. Puromycin, emetine, cycloheximide, streptomycin (Sigma) and chloramphenicol (Parke-Davis) when added were at the concentration of 50 µg/tube. All the tubes contained poly(U) and tRNA (yeast) as shown in Table 1.

90-93% activity with E.coli factors and only about 10-15% activity with cytoplasmic factors. These results are in accordance with previously reported findings in mitochondrial systems of diverse origin (3,9,10).

Discussion

One of the major concerns in preparing mitochondrial specific components from mammalian systems is the contaminating cytoplasmic components(11). Although repeated washing of mitochondrial preparations with buffer containing EDTA markedly reduces the contaminating cytoplasmic specific 80S ribosomal components, in many systems an additional sucrose density centrifugation step is necessary to eliminate the contamination (12). Use of digitonin to wash mitochondria during purification steps presents a rapid and easier way of removing cytoplasmic contamination. At the concentrations used in our studies, digitonin is known to remove the outer membrane (13), that often carries the endoplasmic reticulum which contains bound polysomes. Such digitonin washing method has been shown to minimize cytoplasmic

TABLE 3: FACTOR SPECIFICITY FOR PROTEIN SYNTHESIS BY MITOCHONDRIAL RIBOSOMES

Supernatant Factors from	^{3}H CPM incorporated x 10^{-3}	
	Expt. I	Expt. II
Ehrlich ascites mitochondria	198.6	201.7
Ehrlich ascites cytoplasm	25.7	23.9
E. coli A19	190.8	187.6

Details of in vitro reaction was essentially as described in Table 1 except that instead of S-25, all the tubes contained 1.4 O D 260 nm units of mitochondrial ribosomes and supernatant factors as indicated (125 µg supernatant protein). Each assay tube was also supplemented with poly (U) and tRNA as described in Table 1.

specific rRNA contamination in mitochondrial fractions from Xenopus (14) and Ehrlich ascites tumors (6). Further, digitonin was successfully used to purify rat liver mitochondria yielding mitoribosomes essentially free of cytoplasmic components (15). In accordance with these observations, the S-25 fraction prepared from digitonin washed mitochondria in the present studies is essentially free of cytoplasmic specific ribosomes and rRNA. Considering the limited sensitivity of the techniques used, a complete absence of cytoplasmic specific particles in the S-25 extract cannot be ensured. Nevertheless, even if such contaminating particles are present in the extract, they are less than 0.5% of the mitochondrial components isolated.

Mitochondrial specific ribosomes and their associated RNA's have been studied in a wide variety of animal cells (see ref. 7). Most mitochondrial ribosomes from animal sources sediment as 55-60S particles and contain subunits of 28-31S and 38-42S (see ref. 7 for details). Further, under non-denaturating conditions, rRNA components from mammalian and amphibian sources migrate as 20-21S and 12-14S peaks (14,16,17) on poly acrylamide gels. In this respect, results obtained with Ehrlich ascites mitochondria agree with previously reported results in various animal systems.

The S-25 prepared from digitonin washed mitochondria is highly active in

poly(U) directed poly phenylalanine synthesis (Table 1). The activity is dependent on the addition of ATP and GTP, and phosphate generating systems. Further, the system also requires the addition of tRNA for complete activity. A similar tRNA dependence has been reported for the in vitro system prepared from the cytoplasm of Crebs II ascites (5) and Ehrlich ascites cells. Although initial results (not reported here) indicate that the present mitochondrial system does not discriminate between tRNA from yeast, E.coli or Ehrlich ascites cytoplasm, the tRNA specificity has to be checked using natural mRNA's from mitochondria and other sources.

The activity of in vitro mitochondrial system from Ehrlich ascites tumors is inhibited by chloramphenicol, Puromycin and Streptomycin antibiotics, though to varied degrees. Chloramphenicol and Puromycin, both inhibit the activity by 65-75%, while streptomycin is less effective. The sensitivity of this poly(U) directed in vitro system to streptomycin is more like the effects observed in an in vitro system from chick oviducts (18) rather than a sevier inhibition seen in a mitochondrial polysomal system from E.gracilis (19). Further, emetine and cycloheximide which are known to be the inhibitors of 80S type ribosomes are ineffective on the present mitochondrial system. For optimum activity, the mitochondrial ribosomes require the specific supernatant factors from mitochondria. Similar fraction from Ehrlich ascites cytoplasm is less than 15% active. These results are in direct agreemtn with data obtained with Neurospora (20), Xenopus (10) showing a specific requirement for mitochondrialsupernatant factors for activity. The only exceptional case is E.gracilis mitochondrial system which is significantly active with the cytoplasmic factors (21). However, as observed in all the above systems, the Ehrlich ascites mitochondrial system is almost completely active with E.coli supernatant factors.

Under optimum conditions, the present mitochondrial systems is as active as a highly sensitive in vitro system from Ehrlich ascites cytoplasm prepared by the method of Aviv et al (5) which can efficiently translate hemoglobin mRNA. Recent reports from our laboratory (6,22) as well as others (16,23,23) have shown the presence of poly(A) in mitochondrial associated RNA. Although there is a dis-

agreement on the genetic origin of the poly(A) containing RNA's (14,23,24), there is no doubt that these RNA's are involved in intramitochondrial translation (16). In contrast to the "eukaryotic-nature" of at least some of the mRNA's in mitochondria, the mitochondrial specific translation system resembles bacterial systems with respect to its sensitivity towards antibiotics, initiator tRNA, and initiation/elongation factors, etc. (1,2,3,9,20). It would, therefore, be interesting to test the fidelity of translation of various prokaryotic and eularyotic mRNA's in this highly active mitochondrial system.

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