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Protein Synthesis *in vitro* in a System from Plant Mitochondria

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A mitochondrial system from 48h-germinating seeds of *Vigna sinensis* (Linn.) Savi is capable of incorporating L-[U-^{14}C]valine into proteins and is practically insensitve to cycloheximide, but highly sensitive to chloramphenicol and fusidic acid, a potent inhibitor of peptide-chain elongation factor. A system consisting of mitochondrial S-100 fraction and ribosomes from the same source and other cofactors is capable of polyphenylalanine synthesis and behaves similarly with respect to these inhibitors.

Some features of the mitochondrial protein-synthesizing system resemble those of bacterial systems (Eisenstadt & Brawerman, 1964; Kuntzel & Noll, 1967; Smith & Marcker, 1968; Leis & Keller, 1970; Richter & Lipmann, 1970; Lucas-Lenard & Lipmann, 1971).

Fusidic acid, a potent inhibitor of peptide-chain elongation factor G of both prokaryotic and eukaryotic systems, has been reported to have no effect on mitochondrial elongation factor G in *Neurospora crassa* (Kuntzel *et al.*, 1971). However, Richter *et al.* (1972) have demonstrated that bacteriophage-T_3-DNA-directed synthesis of some bacteriophage-specific enzymes is inhibited by fusidic acid in a protein-synthesizing system from yeast mitochondria. In our study with germinating seeds of *Vigna sinensis* (Linn.) Savi, fusidic acid was found to inhibit both L-[U-^{14}C]valine incorporation by intact mitochondria and L-[U-^{14}C]phenylalanine incorporation into polypeptide by a system isolated from the mitochondria.

Methods

Preparation of mitochondria. Seeds of *Vigna sinensis* (Linn.) Savi were germinated in the dark for 48h (Das *et al.*, 1964), and the embryos were separated from the cotyledons. The embryos were chilled rapidly and homogenized by grinding with sea-sand. The homogenate was extracted with 6vol. of ice-cold medium A (0.25M-sucrose–10mM-Tris–HCl buffer, pH7.4) and centrifuged at 1000*g* for 10min to remove nuclei and cell debris. The supernatant was centrifuged at 10000*g* for 40min. The sediment was resuspended in medium A, and after a clarification run at 2000*g* for 10min the mitochondria were sedimented by centrifuging at 10000*g* for 20min.

The mitochondria were further purified on a discontinuous sucrose density gradient consisting of 0.75M-sucrose, 1.0M-sucrose and a cushion of 2.4M-sucrose (Richter & Lipmann, 1970). The mitochondria were collected in the 1M-sucrose layer, adjusted to approx. 0.25M-sucrose and sedimented by centrifugation at 15000*g* for 20min.

Preparation of mitochondrial ribosomes. Mitochondria isolated as described above were suspended in medium B (10mM-$MgCl_2$–20mM-Tris–HCl buffer, pH7.4) to a concentration of 3mg of protein/ml and lysed by the addition of 0.05vol. of a 20% (w/v) Triton X-100 solution in medium B. The ribosomes were sedimented at 105000*g* for 2h. The ribosomal pellet was suspended in medium B (Richter & Lipmann, 1970).

Preparation of mitochondrial S-100 fraction. The S-100 fraction from mitochondria was prepared according to the method of Richter *et al.* (1972) in medium C (1mM-dithiothreitol – 20mM-Tris – HCl buffer, pH7.4). The S-100 supernatant proteins were precipitated with 43g of $(NH_4)_2SO_4$/100ml of the solution. Supernatant protein was adsorbed on a DEAE-cellulose column (1.2cm×25cm) pre-equilibrated with the same buffer, and protein was eluted with 0.35M-KCl (in medium C). The fractions were pooled, concentrated and dialysed against this buffer.

Intact mitochondria and mitochondrial S-100 fraction and ribosomes were used for protein synthesis *in vitro*.

Results and discussion

Table 1 shows that L-[U-^{14}C]valine is actively incorporated into proteins by mitochondria even in the absence of externally added ATP and its generating system, indicating the presence of a sizable nucleoside triphosphate pool within the mitochondria. Preincubation with cycloheximide (50μg/ml) did not affect the incorporation to a significant extent, indicating that the mitochondrial preparation contained very little cytoplasmic contamination. Fusidic acid inhibited valine incorporation by nearly 50% with as low a concentration as 20μg/ml; a higher concentration (80μg/ml) gave greater inhibition (80%).

Table 2 summarizes the effects of cycloheximide

Table 1. *Incorporation of* L-[*U*-^{14}C]*valine into proteins by mitochondria from 48 h-germinating seeds of Vigna sinensis and the effect of some inhibitors on the process*

Results are expressed as means ± S.D. of five experiments. The complete incubation system contained, in a total volume of 1 ml, 1 μmol of ATP, 3 μmol of phosphoenolpyruvate, 10 μg of pyruvate kinase, 5 μmol of $MgCl_2$, 250 μmol of sucrose, 50 μmol of Tris–HCl buffer, pH 7.4, 20 μmol of potassium phosphate buffer, pH 7.4, L-[U-^{14}C]valine (2.3×10^4 c.p.m.; specific radioactivity 35 Ci/mol) and 4–4.5 mg of mitochondrial protein. The incubation was carried out for 2 h at 37°C with constant shaking. The incubation was stopped by the addition of 0.3 ml of 30% (w/v) trichloroacetic acid. For the measurement of radioactivity protein was processed according to the Stachiewicz–Quastel procedure as described by Dube *et al.* (1972). Radioactivities were measured in a Nuclear–Chicago gas-flow counter.

System	Incorporation (c.p.m./mg of protein)
Complete	5351 ± 186
Plus cycloheximide (50 μg)	5022 ± 170
Plus chloramphenicol (50 μg)	2245 ± 125
Plus fusidic acid (20 μg)	2621 ± 90
Plus fusidic acid (40 μg)	1784 ± 75
Plus fusidic acid (80 μg)	1052 ± 61
* Plus fusidic acid (80 μg)	1189 ± 65

* Preincubated with 50 μg of cycloheximide/ml.

Table 2. *Phenylalanine incorporation into polypeptide by a system from mitochondria isolated from 48 h-germinating seeds of Vigna sinensis and the effect of some inhibitors on the process*

Results are expressed as means ± S.D. of five experiments. The complete incubation system contained, in a total volume of 100 μl, 650 μg of mitochondrial ribosomes, 600 μg of mitochondrial supernatant protein, 100 μg of poly(U), 8 μmol of NH_4Cl, 1.2 μmol of $MgCl_2$, 5 μmol of Tris–HCl buffer, pH 7.4, 0.1 μmol of dithiothreitol, 2 μmol of phosphoenolpyruvate, 0.2 μmol of ATP, 0.05 μmol of GTP, 100 μg of stripped tRNA from *Escherichia coli* and L-[U-^{14}C]phenylalanine (1.5×10^4 c.p.m.; specific radioactivity 30 Ci/mol). The incubation was carried out for 2 h at 37°C with constant shaking. Incorporation of phenylalanine into mitochondrial ribosomal protein was measured from radioactivity incorporated in hot-trichloroacetic acid-insoluble protein (Conway & Lipmann, 1964). Radioactivities were measured in a Nuclear–Chicago scintillation counter.

System	Incorporation (c.p.m./mg of mitochondrial ribosomal protein)
Complete	7054 ± 241
Minus purified S-100 protein	520 ± 46
Minus ribosomes	Negligible
Plus cycloheximide (50 μg)	6943 ± 202
Plus chloramphenicol (50 μg)	2120 ± 113
Plus fusidic acid (50 μg)	1400 ± 84
Plus fusidic acid (100 μg)	900 ± 30

and fusidic acid on L-[U-^{14}C]phenylalanine incorporation into polypeptide *in vitro* by a mitochondrial system from *Vigna sinensis*. Cycloheximide, an inhibitor of eukaryotic protein synthesis, had comparatively little effect in the mitochondrial system. Fusidic acid, a potent inhibitor of peptide-chain elongation factor G of both eukaryotic and bacterial systems, inhibited polyphenylalanine synthesis by approx. 80% at a concentration of 50 μg/100 μl. The inhibitory effect of fusidic acid observed in our experiments indicates that, although the protein-synthesizing components of mitochondria isolated from different sources are very similar, minor differences may exist, giving rise to different degrees of sensitivity to the same compound.

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