Theor Appl Genet (1993) 86:312–316

Characterization of protein synthesis by isolated rice mitochondria

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Received October 20, 1992; Accepted November 3, 1992
Communicated by R. Hagemann

Summary. Bacteria-free mitochondria were isolated from aseptically grown, etiolated and green seedlings of both cytoplasmic male-sterile (WA-type) and male-fertile rice (*Oryza sativa* L.). Protein synthesis in these isolated mitochondria was characterized by gel electrophoresis/fluorography and by the incorporation of [^{35}S]-methionine into protein. In the presence of cycloheximide, a set of some 25 discrete polypeptides and an electrophoretically unresolved population were synthesized. This pattern of protein synthesis *in organello* was essentially the same in mitochondria isolated from both male-fertile and male-sterile cytoplasms. Our data does not preclude the possibility, however, that the WA-type CMS possesses a tissue-specific and/or a low abundance mitochondrial protein(s), whose synthesis eluded detection under our experimental conditions. The synthesis of the mitochondria-encoded polypeptides by isolated rice mitochondria was inhibited by chloramphenicol and incompletely inhibited by erythromycin. A minor chloramphenicol-insensitive, cycloheximide-sensitive translation activity was found consistently to copurify with the mitochondria. This activity generated a reproducible electrophoretic profile of a poorly resolved, weakly labelled population of polypeptides and of a few conspicuous polypeptides, including a 42 kDa species.

Key words: Mitochondrial protein synthesis – Cytoplasmic male sterility – *Oryza sativa* L. – Chloramphenicol – Erythromycin – Cycloheximide – Translational activity

Introduction

Because of the structural features of the rice leaf (Kaufman 1955; Dayanandan et al. 1983) and the preferential uptake of amino acids by contaminating bacteria, rice mitochondria prepared by a widely used procedure based on surface sterilization (Forde et al. 1978; Leaver et al. 1983) are not suitable for *in-organello* protein synthesis (Dai et al. 1991). This difficulty may have hindered earlier efforts to correlate the products of protein synthesis in isolated mitochondria with rice cytoplasmic male sterility (CMS). Such correlations, both positive and negative, have been reported for several other higher plants (Forde and Leaver 1980; Bailey-Serres et al. 1986; Dewey et al. 1987; Wise et al. 1987; Makaroff et al. 1989; Nivison and Hanson 1989; Horn et al. 1991; Köhler et al. 1991). A chimeric mitochondrial gene, containing the 5′ portion of an extra ATPase subunit 6 (*atp6*) coding sequence, has already been shown to be associated with the Chinsurah Boro II type CMS of *japonica* rice (Kadowaki et al. 1990).

Having devised a method to prepare bacteria-free rice mitochondria we found that some 25 unique polypeptides were synthesized by isolated organelles of a japonica rice (Dai et al. 1991). The present study was initiated to ascertain whether mitochondria isolated from a stable, widely-cultivated WA-type CMS indica rice (Lin and Yuan 1980; Mignouna et al. 1987) synthesize any aberrant polypeptide that is not produced by mitochondria of an isogenic male-fertile counterpart.

The use of bacteria-free mitochondria, prepared from aseptically cultivated seedlings, has made possible a more rigorous analysis of the protein synthesis process in higher plant mitochondria. Some interesting details are beginning to emerge. For instance, the rate-limiting factor and the preferred energy source of the *in-organello* protein synthesis reaction (Dai et al. 1991), as well as the mitochondrial polysome translation mechanism *in-organello* and *in vitro* (Dai et al., manuscript submitted), have been identified. An additional aim of this work was

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to examine the sensitivity of the mitochondrial protein synthesis process to inhibitors using bacteria-free mitochondria isolated from both etiolated and green higher-plant tissues. An apparent inconsistency in the differential inhibition of organelle protein synthesis by erythromycin, a prokaryotic protein-synthesis inhibitor, has already been reported (Ibrahim and Beattie 1976; Tassi et al. 1983; Newton and Walbot 1985).

The present paper documents the results of our investigations of the inhibitor effect on mitochondrial protein synthesis and of protein synthesis by rice mitochondria isolated from male-sterile and fertile cytoplasms. We found that: (1) polypeptides synthesized by mitochondria isolated from these two cytoplasms did not differ significantly; (2) in addition to chloramphenicol, erythromycin exhibited a partial inhibitory effect on *in-organello* protein synthesis; and (3) a weak chloramphenicol-insensitive, cycloheximide-sensitive translation activity copurified with rice mitochondria.

Materials and methods

Preparation of rice seedlings

The following varieties of rice (*Oryza sativa* L.) were used: the *japonica* fertile cultivar, Tainung-67 (Dai et al. 1991), the *indica* WA-type cytoplasmic male-sterile line, Zhen Shan 97A (Lin and Yuan 1980; Mignouna et al. 1987; Kadowaki et al. 1988), and its isogenic maintainer line Zhen Shan 97B (Mignouna et al. 1987). The treatment of seeds, and the germination and growth of aseptic rice seedlings was the same as that described in Dai et al. (1991).

Isolation of bacteria-free rice mitochondria

Mitochondria were isolated from 9- to 14-day-old rice seedlings 10–15 cm long. The composition and sterilization of solutions/buffers, the pre-treatment of glass-/plasticware, and the procedure for mitochondrial isolation and sucrose-gradient purification have already been described in detail (Dai et al. 1991).

Protein synthesis by isolated mitochondria

Purified mitochondria (approximately 100 μg of mitochondrial protein) were incubated in a 250-μl reaction mixture at 30 °C for 60 min with constant rotatory shaking. The filter-sterilized reaction mixture contained: 10 mM Tricine-KOH (pH 7.2), 5 mM potassium phosphate (pH 7.2), 90 mM KCl, 250 mM mannitol, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, 1 mM GTP, 225 pM [^{35}S]-methionine (approximately 1,000 Ci/mM) and 25 μM of 19 amino acids except methionine; the neutralized energy-generating supplements consisted of either 2 mM ADP and 10 mM sodium succinate or 6 mM ATP, 8 mM phosphocreatine and 4 units of creatine phosphokinase. Five microliters of this reaction mixture was diluted serially and plated on Luria's broth plates to determine the contaminating bacterial content, if any. A 2.5-μl aliquot of the reaction mixture was used to determine the extend of [^{35}S]-methionine incorporation into protein according to Dai et al. (1991).

Analysis of products of protein synthesis in organello

After 60 min of incubation, 2.5 μl of protein-synthesis chasing solution (10 mM methionine and 1.8 mM of 19 other amino acids) was added and the incubation continued at 30 °C for 15 min. The reaction was terminated by adding 1 ml of protein-synthesis stopping solution [0.4 M mannitol, 10 mM Tricine-KOH (pH 7.2), 10 mM methionine, 1 mM EGTA, and 1 mM each of six protease inhibitors: TLCK, TPCK, t-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane, pepstatin A, and bestatin] to the reaction mixture. Mitochondria were pelleted at 12,000 g for 10 min and solubilized in 100 μl of sample loading buffer by heating at 90 °C for 2 min or by quick-freezing in liquid nitrogen and storing at −80 °C for later analysis. The total mitochondrial lysate was analyzed by 15% (w/v) SDS-polyacrylamide (acrylamide : bisacrylamide = 150 : 1) gel electrophoresis according to Laemmli (1970). Gels were stained with Coomassie blue, destained, immersed in 1 M sodium salicylate for 1 h and then dried onto Whatman 3 MM filter paper. The positions of protein size markers in dried gels were inscribed on the filter paper and gels were then exposed to Kodak XAR-5 X-ray film at −70 °C.

Results

Protein synthesis in mitochondria isolated from green seedlings

The wild-abortive type cytoplasmic male-sterile (CMS-WA) line, Zhen Shan 97A, and its maintainer line, Zhen Shan 97B, are of the *indica* variety. The texture, silica content and surface characteristics of their seedlings are distinct from those of the *japonica* variety used in our previous work (Dai et al. 1991). We found that mitochondria of the *indica* variety still had to be isolated from aseptically-cultivated seedlings in order to eliminate the bacterial contamination problem.

Mitochondria isolated from green *indica* rice Zhen Shan 97B seedlings synthesized a population of products not well resolved by gel electrophoresis and some 25 discrete polypeptides each of which exhibited defined electrophoretic mobility and relative abundance (Fig. 1, lane 1). This pattern of protein synthesis *in-organello* was the same for mitochondria isolated from etiolated seedlings (Fig. 2, lane 5). Mitochondria isolated from green Zhen Shan 97B seedlings, like those isolated from TN67 etiolated rice seedlings (Dai et al. 1991), utilized ADP and succinate more efficiently than did the membrane-independent ATP regenerating system (compare Fig. 1, lanes 3 and 1). With the introduction of different energy supplements, some minor variations in the relative abundance of certain discrete polypeptides was observed.

Effect of protein synthesis inhibitors

As visualized by autoradiographic imaging, protein synthesis by mitochondrial samples isolated from green (Fig. 1, lane 2) and from etiolated seedlings (Fig. 2, lane 2) was inhibited markedly by chloramphenicol (CAP). This inhibition was, however, not complete as shown by

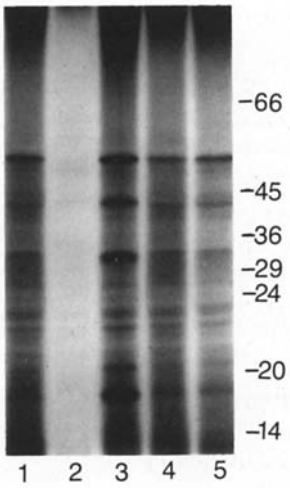

Fig. 1. The effect of protein-synthesis inhibitors on protein synthesis by mitochondria isolated from green Zhen Shan 97B seedlings. Experimental conditions for protein synthesis were as described in Material and methods. Either ADP/sodium succinate or an ATP-regenerating system (ATP/phosphocreatine/creatine phosphokinase) was used as the energy-generating supplement. Each protein-synthesis reaction included the energy-generating supplement and the inhibitor specified. No bacterial colonies were detected by plating 5 µl out of a 250 µl reaction mixture. Lane 1, control, no inhibitor with ATP-regenerating system; lane 2, 100 µM chloramphenicol with ATP-regenerating system; lane 3, control, no inhibitor with ADP/succinate; lane 4, 100 µM cycloheximide with ATP-regenerating system; lane 5, 100 µM erythromycin with ATP-regenerating system

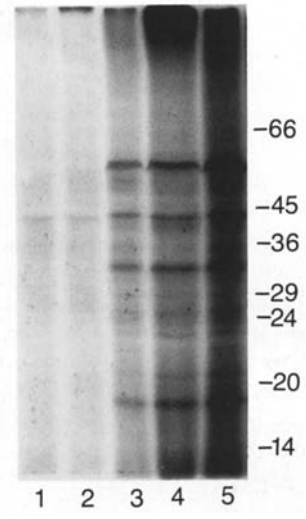

Fig. 2. The effect of protein-synthesis inhibitors on protein synthesis by mitochondria isolated from TN 67 etiolated rice seedlings. Experimental conditions for *in-organello* protein synthesis were the same as in Fig. 1, except that an ATP-regenerating system was used as the energy-generating supplement for all reactions. Lane 1, 200 µM chloramphenicol; lane 2, 100 µM chloramphenicol; lane 3, 100 µM cycloheximide; lane 4, 100 µM erythromycin, lane 5, control, no inhibitor

Table 1. The effects of antibiotics on [^{35}S]-methionine incorporation into mitochondria isolated from etiolated rice seedlings, *Oryza sativa* cv Tainun-67

Antibiotics	Chloramphenicol			Cycloheximide	Erythromycin
Concentration (µM)	100	200	400	100	100
Incorporation (%)	58	30	33	57	65

Mitochondria were isolated from 7–14-day-old etiolated rice seedlings, *Oryza sativa* cv Tainung-67. ATP/phosphocreatine/phosphokinase was the energy supplement for the protein synthesis reaction. Data were averaged from a total of seven experiments

the [^{35}S]-methionine incorporation data. Incorporation of [^{35}S]-methionine into acid-precipitable polypeptides was not further diminished by increasing the CAP concentration above 200 µM (Table 1). A population of not-well-resolved products, including a pronounced polypeptide at 42 kDa, was synthesized by mitochondrial samples in the presence of CAP (Fig. 1, lane 2; Fig. 2, lanes 1 and 2). Apparently some 30% of the incorporated [^{35}S]-methionine resulted from a CAP-insensitive protein synthesis (Table 1).

By autoradiographic evidence, cycloheximide at concentrations from 20 to 100 µM had virtually no effect on protein synthesis in mitochondria isolated from green seedlings (compare Fig. 1, lanes 1 and 4). For mitochondria isolated from etiolated seedlings, the reduction of [^{35}S]-methionine labeling intensity by cycloheximide appeared to be more pronounced for the unresolved population as compared with that of the discrete polypeptides, even through the overall electrophoretic pattern was unchanged (compare Fig. 2, lanes 3 and 5). For mitochondria isolated from etiolated seedlings, the [^{35}S]-methionine incorporation into acid-precipitable polypeptides was reduced by approximately 43% in the presence of cycloheximide (Table 1). Thus, a significant cycloheximide-sensitive translation activity was present in mitochondrial samples isolated from etiolated rice seedlings.

Erythromycin also exhibited an apparent inhibitory effect on protein synthesis by rice mitochondria. In contrast to the effect of cycloheximide, the extent of this erythromycin inhibition appeared to be the same for mitochondria isolated from both green and etiolated seedlings (compare Fig. 1, lanes 1 and 5; compare Fig. 2, lanes 4 and 5). Similar to cycloheximide, erythromycin did not alter the overall electrophoretic pattern of [^{35}S]-methionine-labeled polypeptides, but effected a general decrease in the labeling intensity of the polypeptide products. The [^{35}S]-methionine incorporation data (Table 1) showed that the inhibition amounted to approximately 35% of the control (Table 1).

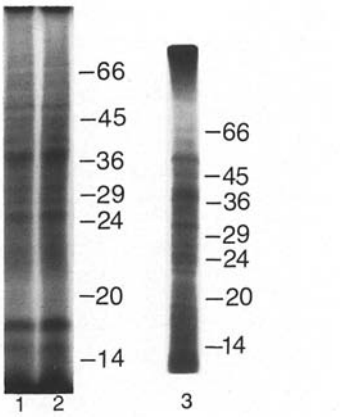

Fig. 3. Electrophoresis profiles of *in-organello* synthesized by Zhen Shan 97A and Zhen Shan 97B. Mitochondria were prepared from etiolated rice seedlings. The data presented here were selected from one of three independent experiments performed under the same *in-organello* protein synthesis conditions and run separately in 15% SDS-polyacrylamide gels. Lane 1, mitochondria isolated from Zhen Shan 97A, a WA-type CMS line; lane 2, mitochondria isolated from Zhen Shan 97B, a fertile maintainer line for Zhen Shan 97A; lane 3, mitochondria isolated from Tainung 67, a fertile *japonica* variety

Protein synthesis by mitochondria isolated from the WA-type CMS line

Polypeptides synthesized by etiolated mitochondria of fertile *indica* and *japonica* rice did not differ significantly, although minor differences in the relative amount of certain species was observed (compare Fig. 3, lanes 2 and 3). A careful analysis of several autoradiograms revealed that the total number and apparent molecular weight of polypeptides synthesized by isolated mitochondria was essentially the same for these two types of male-fertile rice. The autoradiographic images of most polypeptides synthesized by isolated *indica* rice mitochondria were not as sharp as those of their *japonica* counterparts, even though the protein-synthesis activity did not differ significantly between the two types.

Isolated mitochondria of the WA-type CMS line and of its maintainer line each synthesized a set of some 25 discrete polypeptide species (Fig. 3, lanes 1 and 2). Except for minor variations in the relative labelling intensity of certain species, there are no apparent differences in electrophoretic pattern between these two species (Fig. 3). Therefore, irrespective of the fertility of the WA-type CMS line, significant qualitative differences in the synthesis of mitochondria-encoded polypeptides by isolated rice mitochondria were not detected under our experimental conditions.

Discussion

In the present study, neither the appearance of variant, nor the disappearance of regular, polypeptides was detected among the polypeptide products synthesized by isolated mitochondria from the WA-type male-sterile cytoplasm. In the light of the chimeric mitochondrial gene, containing the 5′ portion of an extra ATPase subunit 6 gene that has been shown to be associated with the Chinsurah Boro II CMS in *japonica rice* (Kadowaki et al. 1990), there are at least a few alternative interpretations to our data. For instance: (1) that the chimeric gene found in Chinsurah Boro II type CMS is not present or not expressed at the seedling stage in the WA-type CMS; (2) that such a chimeric gene or an analogous aberrant gene is present in the WA-type CMS but that the aberrant mitochondrial gene product synthesized by isolated seedling mitochondria was too scarce to be recognized as a discrete polypeptide under our experimental conditions; and (3) that no aberrant mitochondrial protein gene product(s) is involved in WA-type rice CMS.

As expected, CAP inhibited effectively the bulk of polypeptide synthesis by isolated mitochondrial samples. On the other hand, a set of CAP-insensitive polypeptides at low concentration, with a prominent 42 kDa constituent, was consistently detected. Consistent with this CAP-inhibition pattern, cycloheximide was shown to reduce mainly the synthesis of the unresolved population (Figs. 1 and 2). Taken together, these data indicate that the unresolved population contained, in addition to partially degraded and/or incomplete nascent mitochondrial polypeptide chains, a small amount of polypeptide products originating from contaminating cytosolic polysomes that copurified with the mitochondria. Also consistent with this interpretation is the effect of CAP on [^{35}S]-methionine incorporation into acid-precipitable polypeptides. At a CAP concentration as high as 400 μM, some 30% of such incorporation remained (Table 1).

Owing to the fact that most of the cycloheximide-sensitive polypeptides synthesized on contaminating cytosolic polysomes were too short to be fractionated by the gel, but were nevertheless precipitated by trichloroacetic acid, the inhibitory effect of 100 μM cycloheximide was far more significant on ^{35}S-methionine incorporation (Table 1) than on polypeptide synthesis, as shown by autoradiography (Figs. 1 and 2). This is especially true for the mitochondrial samples isolated from green seedlings. It is not immediately obvious why mitochondrial samples isolated from green rice seedlings contained less cycloheximide-sensitive translational activity than their counterpart isolated from the etiolated seedlings (compare Figs. 1 and 2).

Erythromycin was first reported to be a selective inhibitor of tobacco plastid protein synthesis *in vivo* (Tassi et al. 1983). Since then it has been generally inferred that erythromycin has no effect on mitochondrial protein synthesis *in-organello* (Newton and Walbot 1985). By assuming a stringent target specificity for cycloheximide (cytosolic ribosome), CAP (mitochondrial and plastid ribo-

somes) and erythromycin (plastid ribosome), a rough estimate based on [^{35}S]-methionine incorporation (Table 1) would show that the plastids' contribution to cycloheximide-insensitive protein synthesis (35%) surpassed that of the mitochondria (22%). This is certainly not reflected in what was actually observed in Figs. 1 and 2, aside from the fact that mitochondrial samples isolated from etiolated rice seedlings were undoubtedly contaminated by some plastids. To make the mitochondria's contribution twice as much as, or equal to, that of plastids, the partial inhibition of *in-organello* mitochondrial protein synthesis by erythromycin would have to be 22.8 or 57.9%. These coarse estimates, coupled with the autoradiographic data (Figs. 1 and 2), indicate that protein synthesis by isolated rice mitochondria was partially inhibited by erythromycin (at a concentration of 100 μM). A similar conclusion was reported in an earlier study (Ibrahim and Beattie 1976).

Acknowledgements. We thank Drs. H.P. Wu and D.J. Liu for their generous efforts in gathering seeds for use in our present study. This work was supported by research grants from the National Science Council, Republic of China and Academia Sinica (to H.D.) and by a research fund from the University of Chicago (to K.S.C.).

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