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Glyoxysomal malate synthetase is specifically degraded in microbodies during greening of pumpkin cotyledons

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A cDNA clone (pKMS: insert, 1.9 kbp) for glyoxysomal malate synthase was prepared from a cDNA library constructed by poly(A)+ RNA from etiolated pumpkin cotyledons and was recombined into the in vitro transcription plasmid, Bluescript. Malate synthase synthesized in an in vitro transcription-translation system was translocated into isolated glyoxysomes. The translocated malate synthase was protected from digestion by added proteinase K. Malate synthase, a glyoxysome-specific enzyme, could also be translocated into leaf peroxisomes prepared from green pumpkin cotyledons, indicating that the machinery for protein translocation into leaf peroxisomes is similar to that into glyoxysomes. When microbodies prepared from greening pumpkin cotyledons were used in the translocation experiments, the translocated malate synthase was observed to be degraded. This result shows that the glyoxysome-specific enzyme, malate synthase, is degraded in microbodies during the microbody transition from glyoxysomes to leaf peroxisomes.

Protein degradation; Translocation; Malate synthase; Microbody transition; (*Cucurbita* sp.)

1. INTRODUCTION

In some fatty seedlings such as pumpkin and watermelon, glyoxysomes are replaced by leaf peroxisomes during greening of cotyledons [1]. Electron-microscopic immunocytochemical studies have demonstrated that glyoxysome-specific enzymes in pre-existing glyoxysomes are replaced by newly synthesized leaf peroxisome-specific enzymes during this transition [2,3], indicating that glyoxysomal enzymes such as malate synthase and citrate synthase may be preferentially degraded in glyoxysomes while newly synthesized leaf peroxisomal enzymes such as glycolate oxidase and hydroxypyruvate reductase are imported into the microbodies. Here, based on results obtained in in vitro translocation experiments, we report that glyoxysomal malate synthase is indeed rapidly degraded in microbodies undergoing the microbody transition from glyoxysomes to leaf peroxisomes.

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2. MATERIALS AND METHODS

2.1. *Plant materials*

Pumpkin seeds (*Cucurbita* sp. Amakuri Nankin) were germinated under the conditions described in [4].

2.2. *cDNA cloning for malate synthase*

A cDNA clone for malate synthase (pKMS) was isolated by immunoscreening from a cDNA library constructed with plasmid vector, pKEN102, and poly(A)+ RNA of etiolated pumpkin cotyledons by the method of Nakamura et al. [5].

2.3. *In vitro translocation of malate synthase into microbodies*

The insert of pKMS was recombined into the in vitro transcription plasmid, Bluescript (Stratagene), and the recombinant (pBSMS) was used as the template for in vitro transcription. Malate synthase-specific mRNA obtained by in vitro transcription with T_7 RNA polymerase was translated in a rabbit reticulocyte lysate translation system containing [^{35}S]methionine (1000 Ci/mmol) [4]. The translate (120 μl) was diluted with HM buffer (10 mM Hepes-KOH, pH 7.2, 1 mM

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EDTA, 0.3 M mannitol) to 1 ml. Non-radioactive methionine was added to the diluted translate to a final concentration of 20 mM. 70 μl diluted translate was incubated with 35 μl purified microbodies (50 μg protein) and incubated at 20°C. After incubation for 1, 15 and 30 min, aliquots of 30 μl each were withdrawn and 1 μl of 150 μg/ml proteinase K was added (final concentration 5 μg/ml). After incubation for 15 min on ice, the mixture was layered on 400 μl of 10% Percoll-HM buffer and centrifuged at 4800 × g for 4 min using a swinging-bucket rotor to sediment microbodies. The surface of the pellet was rinsed twice with 200 μl HM buffer, and 20 μl SDS sample buffer [6] was added. After boiling for 2 min, the sample was subjected to electrophoresis on a 12.5% polyacrylamide gel under denaturing conditions, the gel being then fluorographed.

2.4. *Preparation of microbodies*

Etiolated pumpkin cotyledons grown in the dark for 4 days (80 g) were homogenized three times with a mixer (Sanyo, SM-G171) for 5 s in PM buffer (20 mM pyrophosphate buffer, pH 7.5, 1 mM EDTA, 0.3 M mannitol). The homogenate was filtered through three layers of cheesecloth and centrifuged at 3000 × g for 15 min. The resulting supernatant was centrifuged at 10000 × g for 20 min. The sediment was suspended in 5 ml HM buffer using a glass homogenizer. The suspension was layered on 28% Percoll-HM buffer and centrifuged at 18000 × g for 30 min. Glyoxysomes sedimented near the bottom under such experimental conditions. The glyoxysomal fraction was collected and diluted with 3 vols HM buffer. After centrifugation at 6000 × g for 10 min, the sediment was suspended with 100 μl HM buffer and used for translocation experiments. Leaf peroxisomes and microbodies in the transition stage were isolated by the method described above from green pumpkin seedlings grown in the dark for 4 days and subsequently in the light for 4 days and from greening pumpkin seedlings grown in the dark for 4 days and subsequently in the light for 2 days, respectively. Glyoxysomes, leaf peroxisomes and microbodies in the transition stage were judged to be highly purified and not significantly contaminated with other organelles such as mitochondria or plastids from the analysis of their marker enzyme activities. Specific activities of fumarase (marker of mitochondria) and ribulose-1,5-bisphosphate carboxylase/oxygenase (marker of plastids) in these microbody preparations were less than 3% of those from total cellular extracts.

3. RESULTS AND DISCUSSION

Malate synthase synthesized in in vitro transcription-translation (fig.1, lane 1) was immunoprecipitable with antibodies against pumpkin malate synthase [7] (fig.1, lane 3), and immunoprecipitation was inhibited by the addition of excess cold malate synthase (fig.1, lane 5). The in vitro transcription-translation product comigrated with the immunoprecipitate of the in vitro translation product using poly(A)$^+$ RNA (fig.1, lane 4), which has the same molecular mass as the purified enzyme [7]. Furthermore, malate synthase activity was detected in extracts of COS cells, a monkey kidney cell line, which had been transfected with pKMS which had been subcloned into the mammalian shuttle vector pKSV-10 (Pharmacia) (not shown). These data suggest that the cDNA clone for malate synthase contains the complete coding region and that malate synthase synthesized by the in vitro transcription-translation system can be effectively utilized in translocation studies. Thus, ^{35}S-labeled malate synthase synthesized in the in vitro transcription-translation system could be tested for its ability to be imported post-translationally into glyoxysomes, leaf peroxisomes and microbodies in the transition stage isolated from pumpkin seedlings.

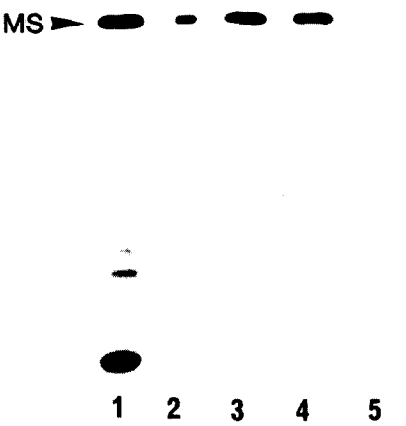

Fig.1. Gel electrophoretic analysis of in vitro translated malate synthase. pBSMS (insert, 1.9 kbp) was linearized with *Bam*HI and the specific RNA was obtained by in vitro transcription with T_7 RNA polymerase. The transcript was capped by the addition of the methylated cap analogue (mGpppG) to the transcription reaction mixture. The RNA was translated in a rabbit reticulocyte translation system in the presence of [^{35}S]methionine and the proteins were immunoprecipitated with specific antibody against malate synthase [7]. Total translated products and immunoprecipitates were subjected to electrophoresis on a 12.5% polyacrylamide gel under denaturing conditions, the gel being then fluorographed. Lanes: (1) malate synthase-specific RNA translation products; (2) immunoprecipitates of in vitro translation products using poly(A)$^+$ RNA from etiolated pumpkin cotyledons [4]; (3) immunoprecipitates of lane 1; (4) mixture of lanes 2,3; (5) immunoprecipitates of lane 1 in the presence of excess cold malate synthase. MS, malate synthase.

The kinetics of uptake of ^{35}S-malate synthase by glyoxysomes and its conversion into a proteinase K-resistant form are shown in fig.2A. Densitometric analysis indicated that approx. 5% of

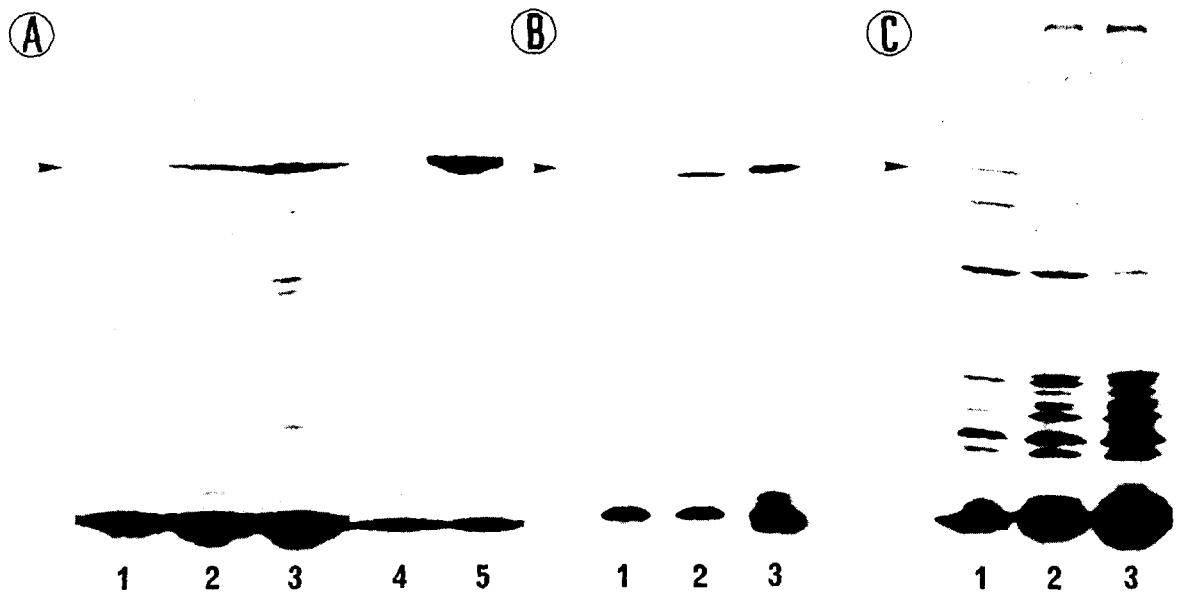

Fig.2. Time-dependent incorporation of in vitro synthesized malate synthase into different forms of microbodies. (A) Glyoxysomes from etiolated pumpkin seedlings, (B) leaf peroxisomes from fully green seedlings, (C) microbodies from seedlings of the transition stage. Arrowhead indicates the band of malate synthase. Malate synthase mRNA was translated for 1 h at 30°C as described in fig.1. In vitro translocation of malate synthase was carried out as described in section 2. Import to (A) glyoxysomes; (B) leaf peroxisomes; (C) microbodies in the transition stage. Lanes: (1) incubation for 1 min, (2) 15 min, (3,4) 30 min in the absence and presence of Triton X-100, respectively; (5) one-tenth of the reaction mixture.

total labeled malate synthase was imported over 30 min under the experimental conditions employed (fig.2A, lanes 3,5). After treatment with Triton X-100, imported malate synthase became proteinase-susceptible (fig.2A, lane 4). These findings indicated that in vitro synthesized malate synthase can be post-translationally imported into glyoxysomes.

Since the enzyme compositions and functions of glyoxysomes and leaf peroxisomes differ from each other [1], it was likely that the two types of microbodies possess different machineries for protein import. However, as shown in fig.2B, time-dependent import of malate synthase into leaf peroxisomes could also be observed, suggesting that the import system into leaf peroxisomes is similar to that present in glyoxysomes.

A striking difference was observed with microbodies isolated from pumpkin seedlings in the transition stage. As the fluorograms presented in fig.2C clearly show, imported malate synthase was rapidly degraded with concomitant formation of lower molecular mass polypeptides. This result indicated that glyoxysomal malate synthase is imported into the microbodies in the transition stage but is degraded rapidly in the organelle. Since practically no degradation of the enzyme was observed in leaf peroxisomes, the protein degradation machinery seemed to operate only transiently during greening.

The results described above can be summarized schematically in fig.3. Glyoxysomal malate synthase molecules are post-translationally imported into leaf peroxisomes and microbodies in the transition stage as well as glyoxysomes. Imported malate synthase molecules are degraded only in the microbodies in the transition stage. Thus, during the microbody transition from glyoxysomes to leaf peroxisomes a degradation system for glyoxysomal enzymes is induced in the microbodies. Further characterization of the degradation system for glyoxysomal enzymes should provide a unique experimental system for clarifying the regulatory mechanism underlying the microbody transition by light at a molecular level.

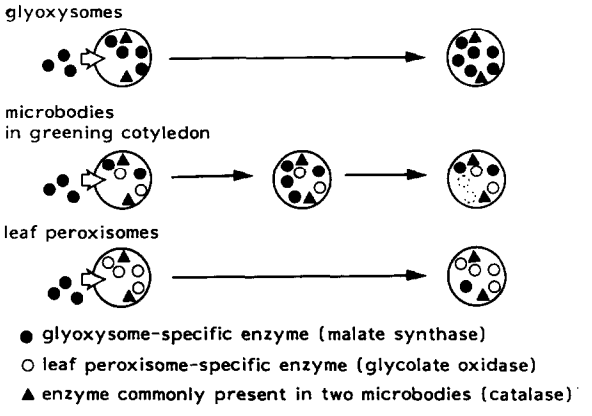

Fig.3. Hypothetical models of import of malate synthase into three different microbodies.

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