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Biosynthesis and intracellular transport of glyoxysomal malate dehydrogenase in germinating pumpkin cotyledons

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Glyoxysomal malate dehydrogenase was synthesized as a larger molecular mass precursor in germinating pumpkin cotyledons. In pulse-chase experiments, the radioactive larger molecular mass precursor (38 kDa) disappeared and was converted to the mature form (33 kDa) of the enzyme. When the radiolabeled cotyledon was fractionated into cytosolic and organellar fractions, the larger molecular mass precursor was first recovered in the cytosolic fraction and then only after a 20 min chase the mature form was found in the organellar fraction. This indicates that the higher molecular mass precursor is synthesized in the cytosol and the processing of the transient precursor is coupled to the transport into glyoxysomes.

Enzyme precursor; Microbody biogenesis; Malate dehydrogenase; Post-translational processing; Protein transport

1. INTRODUCTION

The development of cellular organelles such as chloroplasts, mitochondria and microbodies requires specific import of proteins that are synthesized in the cytosol and subsequently transferred to their respective subcellular destinations. In chloroplasts and mitochondria, most of these organellar proteins are synthesized as larger M_r precursors each containing a cleavable extrapeptide addressed to the postulated organellar translocation machinery [1]. On the contrary, most microbody proteins (including glyoxysomal and peroxisomal ones) are known to be synthesized in a form similar in size to the mature molecule [2,3]. However, there exist some exceptional proteins which are found as higher M_r precursors; i.e., malate dehydrogenase (MDH) in watermelon [4,5] and cucumber [6], catalase in pumpkin cotyledons [7] and 3-ketoacyl-CoA thiolase in rat liver [8]. Further studies are needed to clarify the roles of these exceptional larger M_r precursors in the proteolytic process.

In this communication, we report the in vivo labeling of gMDH from pumpkin cotyledons and its subcellular compartmentation into glyoxysomes, indicating that the processing of the transient precursor is coupled to the transport into glyoxysomes.

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Abbreviations: MDH, malate dehydrogenase; gMDH, glyoxysomal malate dehydrogenase; mMDH, mitochondrial malate dehydrogenase

2. MATERIALS AND METHODS

2.1. *Plant growth and pulse-chase labeling of gMDH*

Pumpkin seeds (*Cucurbita* sp. Amakuri Nankin) were germinated under the conditions described in [9].

Pulse-chase experiments of gMDH using germinating pumpkin cotyledons (5-day-old) were conducted essentially as described in [7].

Subcellular fractionation of the labeled cotyledons was performed according to the chop-

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ping method [9]: half cotyledons were homogenized in 0.5 ml of 150 mM Tricine-KOH (pH 7.5) containing 1 mM EDTA and 13% (w/v) sucrose by chopping with a stainless steel razor blade, and then the homogenate was rapidly centrifuged for 1 min at 600 × *g* in a Kubota microcentrifuge. The supernatant (0.38 ml) was recentrifuged at 16000 × *g* for 2 min, and the precipitates were suspended in 0.38 ml of the above solution and disrupted by sonication. The immunoprecipitation of MDH from the supernatant (cytosolic fraction) and solubilized precipitate (organellar fraction) was carried out separately as described [7].

2.2 *Other methods*

Catalase [9] and fumarase [10] activities were measured spectrophotometrically. Protein was determined according to Lowry et al. [11]. The isolation of mitochondrial and glyoxysomal fractions by sucrose density gradient centrifugation [9] and immunoblotting [12] was performed as reported.

3. RESULTS

3.1. *Immunochemical difference between gMDH and mMDH*

Different MDH isoenzymes are known to be associated with microbodies, mitochondria, and cytosol [5,13]. We have purified gMDH from the glyoxysomal fraction of etiolated pumpkin cotyledons [14]. An antiserum prepared against the enzyme protein was shown to react exclusively with gMDH by Ouchterlony double immunodiffusion and immunoinhibition analyses [14].

Further immunochemical characterization of gMDH and mMDH employing electrophoretic transfer blotting techniques (immunoblotting) is presented in fig.1. It is clear that gMDH reacts with the rabbit antiserum raised against gMDH, whereas mMDH does not, showing that the antiserum used is monospecific to gMDH.

3.2. *Higher* M_r *precursor of gMDH*

We have demonstrated previously that the gMDH synthesized in vitro directed by poly(A)$^+$ RNA prepared from etiolated pumpkin cotyledons in the wheat germ cell-free translation system is a polypeptide of 38 kDa, which is 5 kDa larger than that of mature gMDH (33 kDa) [15].

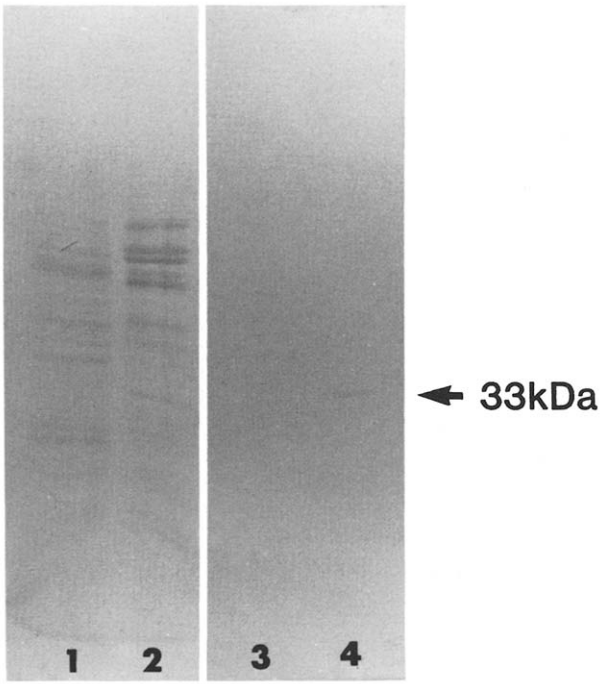

Fig.1. Immunochemical characterization of MDH by electrophoretic transfer blotting techniques (immunoblotting) from mitochondrial and glyoxysomal fractions isolated from germinating pumpkin cotyledons. Lanes: 1,3, mitochondrial fractions; 2,4, glyoxysomal fractions; 1,2, Coomassie brilliant blue staining; 3,4, immunoblotting. Etiolated cotyledons (5-day-old) were homogenized by chopping with a razor blade in homogenizing medium described previously [9]. The homogenate obtained was applied to the sucrose gradient (2 ml of 20% (w/w), 12 ml of linear 30–60% (w/w) containing 1 mM EDTA) and centrifugation was performed at 21 000 rpm for 3 h using a Beckman Spinco SW27-1 rotor with a Beckman L5-50 ultracentrifuge. At the end of centrifugation, both mitochondrial and glyoxysomal fractions were collected by side puncture. The mutual contaminations of other organelles were less than 5% as estimated by assaying marker enzymes (mitochondria, fumarase; glyoxysomes, catalase). The mitochondrial and glyoxysomal fractions containing the same amount of MDH activities were applied to SDS-PAGE. The immunoblotting was performed as described [12].

In order to confirm that this higher M_r polypeptide is actually a precursor of gMDH, we carried out pulse-chase labeling of the etiolated cotyledons (30 min-pulse with [^{35}S]methionine and 20, 60 min-chase with 10 mM unlabeled methionine) (fig.2). First the 38 kDa polypeptide appeared and a

decrease in labeling of the band during 20 to 60 min-chase transition was found, concomitant with an increase in labeling of the 33 kDa polypeptide. These results provide strong evidence that the 38 kDa polypeptide is a precursor of mature gMDH.

3.3. *Intracellular transport of gMDH*

Etiolated cotyledons were labeled with [^{35}S]methionine for 20 min and chased with unlabeled methionine for 20, 60, 120 min. The homogenate was separated into supernatant (cytosolic) and precipitate (organellar) fractions. Incorporation of radioactivity into the tissues increased with time up to 60 min. The recovery of intact glyoxysomes, estimated from catalase activity (marker enzyme of glyoxysome), was 60–70%.

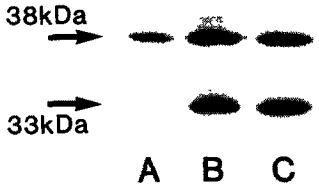

Fig.2. Pulse-chase labeling of gMDH in vivo in germinating pumpkin cotyledons. Lanes: A, 30 min-pulse with [^{35}S]methionine and no-chase; B, 30 min-pulse and 20 min-chase with 10 mM unlabeled methionine; C, 30 min-pulse and 60 min-chase. [^{35}S]Methionine (50 μCi) was administered to the excised half cotyledons from etiolated pumpkin seedlings (5-day-old). After 30 min of incubation at 25°C, the tissue was washed three times with unlabeled methionine (10 mM) and after 0, 20, 60 min-chase homogenized with 1 ml of 100% acetone with a mortar and pestle. The pelleted fraction obtained (8000 × *g*, 5 min) was dissolved in 0.5 ml of 2% SDS containing 10 mM methionine and boiled for 4 min, followed by the addition of Triton X-100 to a final concentration of 4%. After the addition of 1.5 ml of phosphate-buffered saline (pH 7.4) containing 40 μg/ml each of antipain, leupeptin, chymostatin and pepstatin, 10 μl of anti-gMDH antiserum was added and incubation continued for 2 h at 30°C. 100 μl of 10% fixed *S. aureus* cells (Pansorbin from Calbiochem) was added to the mixture, which was allowed to incubate for 3 h at 30°C with occasional stirring. Finally, the cells were precipitated by centrifugation, and after thorough washing twice with phosphate-buffered saline (pH 7.4) containing 1% Triton X-100, 0.2% SDS and 2 mM EDTA and once with distilled water, the immunoprecipitates were subjected to the SDS gel electrophoresis and fluorography as described [7].

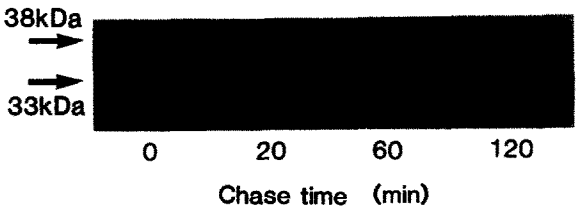

Fig.3. In vivo synthesis and intracellular transport of gMDH in germinating pumpkin cotyledon. Etiolated half cotyledons were incubated with 50 μCi [^{35}S]-methionine for 20 min and chased with 10 mM unlabeled methionine for 0, 20, 60, 120 min. The subcellular fractionation of the labeled cotyledons was performed as described in section 2. The cytosolic (S) and organellar (P) fractions of the tissues were subjected to immunoprecipitation with anti-gMDH antiserum, followed by SDS electrophoresis and fluorography as described in the legend to fig.2.

First, the larger M_r precursor of gMDH (38 kDa) was labeled only in the cytosolic fraction (0 min-chase); then labeling of the 38 kDa polypeptide decreased as the chase period progressed, in parallel with an increase of labeling of mature gMDH (33 kDa) in the organellar fraction (Fig.3). It is likely that the labeling of the 33 kDa polypeptide in the cytosolic fraction is ascribable to release of the mature gMDH from glyoxysomes by disruption of the organelles during the homogenization. Importantly, we demonstrated here that the labeled gMDH species in the organellar fraction is only the mature 33 kDa molecule, indicating that the proteolytic processing engaged in the conversion of the larger M_r precursor to the mature molecule is closely linked with the transport of the polypeptide into the glyoxysomes.

4. DISCUSSION

The reaction in the conversion of the larger M_r precursor of gMDH into the mature molecule is rapid and complete (fig.3) and we were unable to detect the transient larger M_r precursor in glyoxysomes (fig.3). These results are in contrast to the findings concerning the intracellular transport of another higher M_r precursor into microbodies. In rat hepatocytes it has been reported that both labeled higher M_r precursor and mature molecular forms of 3-ketoacyl-CoA thiolase are nearly equally distributed between the cytosolic and organellar fractions [16]. In the case of catalase in pumpkin

cotyledons, whose larger M_r precursor is accumulated in microbodies, it has been found that the processing does not play a role in the import into organelles but does play a role in activation of the enzyme [7,12]. It thus appears that the proteolytic processing of the transient peptide of the larger M_r precursor of gMDH is obligatory for the mechanism of transport into microbodies, which is an exceptional case in the transport system of microbody enzymes into the organelles. However, it cannot be completely ruled out that the gMDH precursor is first transported into the organelles without coupling to proteolytic process, and subsequently processed so rapidly that the larger M_r precursor cannot be detected in the organelles (see fig.3).

Recently Gietl and Hock [17] have reported a 'heterologous' in vitro system for the import of the higher M_r precursor of gMDH from watermelon cotyledons into glyoxysomes from castor bean endosperm, demonstrating that the process of import by the organelle is independent from the energization of the glyoxysomal membrane.

As to the larger precursor of MDH and the processing, Hock [13] has proposed an interesting hypothesis. He focused on the mode of intracellular distribution of MDH with regard to different organelles in watermelon cotyledons. It is known that different MDH isoenzymes are associated with mitochondria (mMDH, 38 kDa), glyoxysomes (gMDH, 33 kDa) and cytosol. From analyses of mMDH and gMDH by immunochemical and pulse-chase experiments, he suggested that the gMDH and mMDH are derived from a common precursor of high molecular mass (41 kDa) and by alternative post-translational processing, transient sequences of different lengths were removed, triggering a different three-dimensional folding of the resulting products. His postulation is based on the results that the immunochemical difference between gMDH and mMDH is abolished under denaturation conditions. However, since we have demonstrated that the gMDH molecule has a different immunochemical response from mMDH before and after denaturation, it is clear that this interesting hypothesis needs to be reassessed.

Recently we have purified the specific mRNA for gMDH by immunoabsorption and the preparation of the cDNA is under way. Further analysis of gMDH using the cDNA should provide useful information on the transport mechanism of microbody proteins.

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REFERENCES

[1] Schmidt, G.W. and Mishkin, M.L. (1986) Annu. Rev. Biochem. 55, 879–912.
[2] Trelease, R.N. (1984) Annu. Rev. Plant Physiol. 35, 321–347.
[3] Lazarow, P.B. and Fujiki, Y. (1985) Annu. Rev. Cell Biol. 1, 480–530.
[4] Walk, D.-A. and Hock, B. (1978) Biochem. Biophys. Res. Commun. 81, 636–643.
[5] Gietl, C. and Hock, B. (1982) Plant Physiol. 70, 483–487.
[6] Riezman, H., Weir, E.M., Leaver, C.J., Titus, D.E. and Becker, W.M. (1980) Plant Physiol. 65, 40–46.
[7] Yamaguchi, J., Nishimura, M. and Akazawa, T. (1984) Proc. Natl. Acad. Sci. USA. 81, 4809–4813.
[8] Furuta, S., Miyazawa, S., Hashimoto, T., Miura, S., Mori, M. and Tatibana, M. (1982) Biochem. Biophys. Res. Commun. 105, 639–646.
[9] Yamaguchi, J. and Nishimura, M. (1984) Plant Physiol. 74, 261–267.
[10] Racker, E. (1950) Biochim. Biophys. Acta 4, 211–214.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Yamaguchi, J., Nishimura, M. and Akazawa, T. (1986) Eur. J. Biochem. 159, 315–322.
[13] Hock, B. (1984) Physiol. Veg. 22, 333–339.
[14] Mori, H., Nishimura, M., Akazawa, T. and Yokota, S. (1986) Plant Physiol., in press.
[15] Nishimura, M., Yamaguchi, J., Mori, H. and Akazawa, T. (1984) Proc. International Cell Biology 1984, The Japan Society for Cell Biology, p. 288.
[16] Miura, S., Mori, M., Takiguchi, M., Tatibana, M. Furuta, S., Miyazawa, S. and Hashimoto, T. (1984) J. Biol. Chem. 259, 6397–6402.
[17] Gietl, C. and Hock, B. (1986) Planta 167, 87–93.