

Rates of *de novo* Synthesis of Malate Synthase and Albumins during the Very Early Phase of Germination

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Malate synthase is synthesized *de novo* in the very early phase of germination. Its molecular and immunological properties do not differ from those of malate synthase from fully developed cotyledons. Radioactive leucine was administered to dry seeds of cucumber, and its incorporation into proteins of cotyledons was examined after 2 days of germination. The specific radioactivity of malate synthase, purified by immunoprecipitation and electrophoresis on polyacrylamide gel, was only 1/20 the average value of the total albumin fraction. The minimal incorporation documented by the comparatively low specific activity of isolated malate synthase is discussed in relation to the large pool of malate synthase already present in dry seeds.

Introduction

During germination of fat-containing seeds a drastic increase of all glyoxysomal enzyme activities is observed. Activities of catalase, isocitrate lyase and malate synthase multiply by a factor of more than 5 between day 1 and 5 of germination [1–3]. In many seeds, enzyme activities could not be detected before day 1 of germination [4, 5], whereas in one case – with cucumber seeds [6] – we were able to demonstrate that all typical glyoxysomal proteins were already present, in small amounts, in dry seeds prior to germination.

In connection with the preceding paper [7] we intended to compare the presence or formation of glyoxysomal proteins in subsequent developmental steps: a) during seed ripening, and b) in the early phase of seed germination. Virtually all investigations on the biosynthesis of glyoxysomal components have been done, so far, with germinating seeds at a subsequent stage, from day two onwards.

In this paper we present evidence that malate synthase was synthesized *de novo* already during the first two days of germination. Nevertheless, the amount of malate synthase formed was much smaller than the amount of other albumins, although this enzyme constitutes about 0.5% of the soluble proteins of the cotyledons at this stage.

Abbreviations: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TCA, trichloroacetic acid.

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Materials and Methods

Application of radioactive precursors

One hundred dry cucumber seeds (*Cucumis sativus*) were surface sterilized with 0.05% hypochloride, washed thoroughly with sterile water and incubated for 18 h at 27 °C simultaneously with 1.0 mCi [$1-^{14}\text{C}$] acetate (60.2 mCi/mmol) and 1.0 mCi [$4,5-^{3}\text{H}$] leucine (60 Ci/mmol). Then, the seeds were germinated on moistened filter paper at 27 °C for 30 h.

Isolation of albumins, malate synthase, globulins and protein bodies

Fifty pairs of cotyledons were homogenized in 5 ml buffer consisting of 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 10 mM MgCl_2 , and 5 mM glyoxylate. The homogenate was centrifuged at $20\,000 \times g$ for 30 min. The fat layer was carefully removed and the remaining supernatant fraction centrifuged at $100\,000 \times g$ for 30 min. The supernatant obtained after this second centrifugation contained the albumins.

The pellet of the $20\,000 \times g$ -centrifugation was suspended in 5 ml of 2 M NaCl, in 50 mM Tris-HCl, pH 7.5, for 1 h at room temperature and subsequently centrifuged at $20\,000 \times g$ for 30 min. The supernatant fraction was taken as globulins. *Malate synthase* was purified from the albumin fraction by immunoprecipitation.

The other 50 pairs of cotyledons were homogenized in glycerol using a waring blender. The crude homogenate was centrifuged at $40\,000 \times g$ for 30 min



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according to Yatsu and Jacks [8]. The protein body-pellet was resuspended in 4 ml sucrose (30%, w/w) prepared in 50 mM Tris-HCl, pH 7.5, and layered onto a gradient. For this purpose, a gradient was prepared from 8 ml 62% sucrose, 6 ml 55% sucrose, 6 ml 50% sucrose, 7 ml 45% sucrose, and 7 ml 40% sucrose. All sucrose solutions (percent, w/w) were prepared in 50 mM Tris-HCl, pH 7.5. Centrifugation was for 2.5 h at 27 min⁻¹ (Beckman SW-27 rotor).

Determination of specific radioactivities

In the case of total protein, precipitation was with cold TCA (7%). The pellets were washed twice and solubilized in soluene TM 100 (Packard). Specific radioactivity of malate synthase was determined corresponding to a procedure reported by Gustavson *et al.* [9]. A malate synthase-antibody complex was dissolved in sodium dodecyl sulfate (2%) and subjected to electrophoresis in the presence of mercaptoethanol and sodium dodecyl sulfate. For quantitation of the protein on the gel, the destained gel was scanned at 620 nm using an Isco gelscanner. The protein was determined from the densitometer scan by comparison with control scans of varying amounts of purified malate synthase. Within a range of 0.01–0.1 mg malate synthase the intensity of stain was shown to be strictly proportional to the amount of protein. The intensity was determined from the area under peaks. These areas were determined by cutting out the peaks and weighing the papers. Subsequent to the determination of protein, the slab gel was cut into 1.5 mm pieces and the radioactivity determined [7, 10].

Other methods

Gradient centrifugation for preparation of microbodies, electrophoretic analysis, immunoprecipita-

tion, determination of radioactivity and protein were carried out as described in the preceding paper [7]. Enzymes were assayed according to established methods [7, 11]. Autoradiography was carried out by the method of Bonner and Laskey [12].

Results

A series of experiments was performed aimed at the incorporation of radioactive amino acids into malate synthase. Results indicated that, during the early stage of germination, only very small amounts of malate synthase were *de novo* formed. More information on protein synthesis might be drawn from comparison, on the basis of rate of incorporation, of malate synthase with other proteins or classes of proteins. [³H]Leucine was used as precursor, and [¹⁴C]acetate was administered simultaneously; the ratio ³H/¹⁴C was 1.01. The rationale for the way incorporations were carried out was based on the idea that an artificially labelling of proteins, which would mimic a *de novo* synthesis, should be recognized. Leucine reportedly can be degraded to acetic acid which might modify an already existing protein. Accordingly, a ratio ³H/¹⁴C higher than one in the product must be indicative of a biosynthesis from leucine without its fragmentation to acetic acid prior to incorporation.

After a two day-period of incorporation, cotyledons were removed from roots and hypocotyls. Albumins and globulins were prepared from either tissues.

Incorporation of radioactivity into proteins of the albumin fraction

The comparison of specific activities, summarized in Table I, revealed that the radioactivity of the pre-

Table I. Comparison of labelling of albumins and globulins.

[³H]Leucine and [¹⁴C]acetate were administered to dry seeds. Seeds imbibed by the solution containing the radioactive components were germinated for 2 days. Albumins and globulins from 50 cotyledons were separated by differential extraction. Malate synthase was purified by immunoprecipitation. For reason of comparison, albumins from roots (and hypocotyls) were also prepared.

| Fraction | Source | Protein [mg] | Radioactivity [nCi] | Specific Radioactivity [nCi/mg] | Ratio ³ H/ ¹⁴ C |
|-----------------|------------|-----------------|------------------------|---------------------------------------|--|
| Albumin | cotyledons | 30.0 | 4 460 | 148.7 | 3.1 |
| Malate Synthase | cotyledons | 0.06 | 0.40 | 6.7 | 1.8 |
| Globulin | cotyledons | 46.5 | 640 | 13.7 | 1.4 |
| Albumin | roots | 10.4 | 30 700 | 2 952.9 | 3.0 |

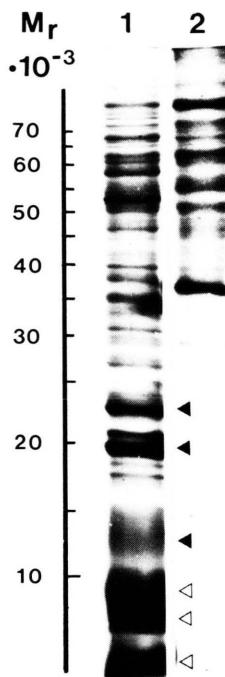


Fig. 1. Electrophoretic separation of labelled albumins from 2 day old cotyledons. Albumins were precipitated with TCA and electrophoresed in a 10% polyacrylamide slab gel in the presence of sodium dodecyl sulfate. The gel was stained (lane 1), dried and autoradiographed for 6 days (lane 2). The subunits of the dry seed albumin and the globulin digestion product is marked with ▲ and ▼, respectively.

cursor was predominantly transferred to the meristematic tissues, in roots and hypocotyls. The albumins of these newly cells were highly labelled. The specific activity of this protein fraction was 20 times as high as the average value obtained with the albumins of the cotyledons (Table I).

A number of proteins within the albumin fraction from cotyledons exhibited specific activities several times higher than given by the average value. This could be verified by comparing the protein patterns with the radioactivity distribution illustrated by autoradiography. As can be seen from Fig. 1, the heavily labelled bands of the albumin fraction were not always the most dominant bands stained for proteins. The dominant proteins with M_r of approximately 7000–9000 had previously been shown to be the most prominent albumins of dry cucumber seeds [6].

Furthermore, Reilly *et al.* [13] substantiated that the first step of globulin breakdown in pumpkin seeds yielded a water soluble digestion product. With-

in cucumber seeds, this digestion product comprises proteins with M_r 23000, 20000 and 13000 (Fig. 1), if reduced with mercaptoethanol prior to electrophoresis (unpublished results). These albumin subfractions, dry seed albumins and globulin digestion products, are shown to remain unlabelled (Fig. 1). Therefore, one can assume that the specific radioactivity of the peptide bands in the M_r range > 35000 were considerably higher than expressed by the average value of the total fraction presented in Table I.

Incorporation of radioactivity into malate synthase and other proteins of the glyoxysomal membrane

Malate synthase, a constituent of the albumin fraction, was purified by immunoprecipitation and

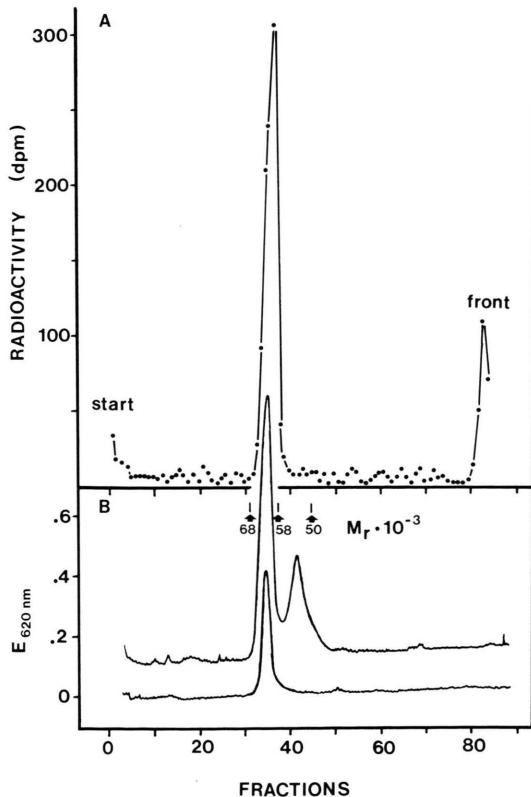


Fig. 2. Profiles of radioactivity and protein staining of an immunoprecipitation obtained from albumin fraction using antibodies raised against glyoxysomal malate synthase. The lower most curve indicates the position of authentic malate synthase; the other curve shows bands, malate synthase and heavy chain of globulins, developed after electrophoresis of the immunoprecipitation. Experiments described elsewhere [17] have shown that the antibodies used here precipitate not only glyoxysomal malate synthase but also cytoplasmic malate synthase.

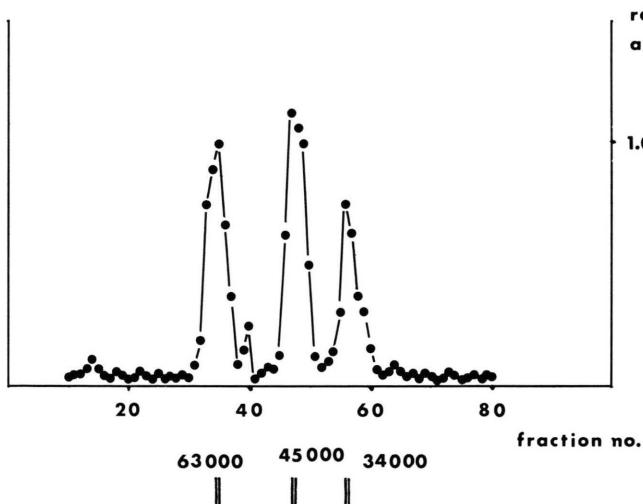


Fig. 3. Electrophoretic analysis of labelled glyoxysomal membranes. 25 Dry seeds were incubated with 0.5 mCi [^3H]leucine for 2 d. Homogenates were prepared and fractionated by isopycnic density gradient centrifugation. Glyoxysomes were removed and separated into a matrix and a membrane fraction [11]. The membrane preparation was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Malate synthase (subunit M_r 63000), citrate synthase (subunit M_r 45000) and malate dehydrogenase (subunit M_r 34000).

analyzed by electrophoresis (Fig. 2). The protein stain showed, besides the heavy chain of immunoglobulin at M_r 54000, only one sharp band at M_r 63000. Thus, malate synthase present in two day old cotyledons exhibited exactly the same subunit M_r as glyoxysomal malate synthase of five day old cotyledons. Although Fig. 2 illustrates the labelling of purified malate synthase, its specific activity was surprisingly low (Table I). The average value within the albumin fraction was 22 times higher than specific activity of this distinct constituent. The actual difference in amino acid incorporation between the most intensively labelled proteins in the albumine fraction (Fig. 1) and malate synthase may be rendered, however, by a factor of more than 50. Moreover, the specific activity of the peptide bands, in the albumin fraction in the M_r range 60000–65000 (Fig. 2) was estimated to be higher than 500 nCi/mg protein. The specific activity of the M_r 63000 zone of malate synthase, determined independently, was 6.7 nCi/mg. This rules out that malate synthase could have been visualized in the track (Fig. 2 B). Autoradiography permitted a survey of the most prominent bands only.

The following experiment was carried out with 2 day old cotyledons which had received [^3H]leucine from day 0 (= dry seed) onwards. The cells were homogenized and microbodies were isolated by isopycnic density centrifugation. From the membranes, malate synthase, citrate synthase and malate dehydrogenase were solubilized, and separated by poly-

acrylamide gel electrophoresis in the presence of mercaptoethanol and sodium dodecyl sulfate.

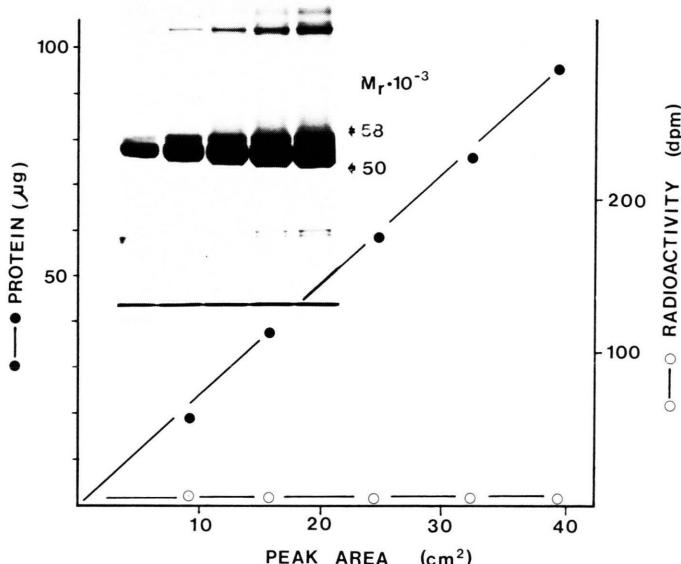
Fig. 3 showing the radioactive profiles on the gel indicates a) that the malate synthase of the glyoxysomal membrane was labelled, and b) that citrate synthase and malate dehydrogenase were equally well labelled.

Was radioactivity incorporated into the main globulins of protein bodies?

In cotyledons, the specific radioactivity of globulins was low compared with the albumin fraction, but even higher than the value determined for malate synthase (Table I). Thorough examination of the main globulin, *i.e.* the globulin of dry seeds (M_r = 54000) described previously [6], proved that this component was not labelled. Protein bodies, prepared by grinding and centrifugation in glycerol, were disrupted with water. This treatment released a protein crystalloid. The crystalloid behaved, upon density gradient centrifugation, like the protein body crystalloid of castor bean seeds [14] and banded at a density of 1.29 kg/l (data not shown).

We analyzed, by gel electrophoresis (Fig. 4), increasing amounts of the isolated crystalloid. The analysis showed that the crystalloid was composed of the main globulin found in dry seeds [6]. It exhibited, in the absence of mercaptoethanol, a M_r of 54000. Even at application of very high protein concentrations we failed to detect radioactivity (< 50 dpm/mg) within the protein bands (Fig. 4).

Fig. 4. Analysis of a crystalloid from protein bodies by gel electrophoresis in the presence of sodium dodecyl sulfate. Increasing amounts of protein (0.02–0.1 mg from left to right) were electrophoresed in a 8% slab gel in the absence of mercaptoethanol. After destaining the gels were scanned, and the peak area of the protein bands at M_r 54000 was determined. Subsequently, the radioactivity of the corresponding protein bands was determined.



Discussion

In cotyledons, albumins in contrast to globulins were effectively labelled even in the very early phase of germination. The pronounced difference in specific activity between albumins of cotyledons and roots reflects the fact that in cotyledons a considerable pool of endogenous protein interferes with the pool of precursors. No glyoxysomal component so far studied was labelled to such an extent that could be assumed from the findings that malate synthase constitutes 0.3–1% of the soluble protein in cucumber cotyledons. We found, moreover, that the radioactivity incorporated into other proteins solubilizable from the glyoxysomal membrane did not exceed the one attributable to malate synthase.

Most surprisingly, the specific activity of this malate synthase was only 1/20 of the value which was determined as average for the total albumin fraction of cotyledons. Compared with the highly labelled albumins with subunit M_r 80 000, 63 000, 55 000,

37 000 (Fig. 1), the factor illustrating the different intensities of labelling might be even higher than 50.

We found that the enzyme activity of malate synthase was increased by a factor 2 between day 0–2 of germination. When malate synthase was present in dry seeds [6, 15], and if we assume that a high proportion of this malate synthase is not associated with glyoxysomes [16–18], we could interpret the findings by proposing the hypothetical scheme: During assembling of malate synthase in the glyoxysomal membrane a presently unknown process of rearrangements takes place which is responsible for the increase in hydrophobicity of the protein and for the multiplication of specific enzyme activity. Both findings, that this rearrangement is a disaggregation of a highly aggregated malate synthase, a process which parallels the drastic increase in specific activity, and the low rate of the *de novo* synthesis reported here, support the hypothesis.

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