THE APPEARANCE OF SEVERAL MALATE SYNTHASE-CONTAINING CELL STRUCTURES DURING THE STAGE OF GLYOXYSOME BIOSYNTHESIS

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1. Introduction

There is now compelling evidence that malate synthase is a membrane-bound, peripheral enzyme of glyoxysomes [1–3]. This enzyme together with two other peripheral and two matrix proteins provides the catalytic basis for the function of the glyoxylate cycle during the germination of plant seedlings. Findings such as the demonstration of morphological continuity between the endoplasmic reticulum and glyoxysome [4], the similarities in the phospholipid composition of their membranes [5], the phospholipid flow in vitro from endoplasmic reticulum to glyoxysomes [6] indicate that glyoxysome biosynthesis may take place by segmenting vesicles from the dilated cisternae of endoplasmic reticulum. Similarity in peptide pattern of microsomal and glyoxysomal membranes [7,8] also favours this model. Hence, a developmental stage characterized by the appearance of vesicles or a form of endoplasmic reticulum which contains malate synthase may be thought to precede glyoxysome formation.

Here we provide evidence that, in the phase of glyoxysome biosynthesis, malate synthase is also associated with structures distinguishable from endoplasmic reticulum or glyoxysomal membranes.

2. Materials and methods

2.1. *Plant material and preparation of homogenates*

Seeds of *Cucumis sativus* (Chinesische Schlangengurken) were surface-sterilized with NaOCl, thoroughly washed with sterile water and germinated at 27°C on vermiculite.

Cotyledons were homogenized with a scalpel for 30 min in the presence of grinding medium. The latter contained 150 mM Tris–HCl (pH 7.5), 10 mM KCl, 1 mM EDTA and 13% (w/w) sucrose. The crude homogenate was squeezed through two layers of Miracloth.

2.2. *Centrifugation procedures*

For the zonal centrifugation of the homogenate, sucrose gradients ranging from 15–60% (w/w) were constructed in such a way that the gradient in the region 15–30% sucrose was very shallow and comprised 60% total volume. The technique and the equipment used was as in [9].

The recentrifugation of the heavy membrane fraction was carried out in a Beckman-14-Ti zonal rotor. The following solutions (in 50 mM Tris–HCl, pH 7.5 and 1 mM EDTA) were pumped into the rotor: 50 ml 23% sucrose, 100 ml 30% sucrose, 50 ml sample (brought to 35% sucrose), 250 ml 40% sucrose, 70 ml 43% sucrose and 48%, 55%, 60% sucrose (40 ml each).

A corresponding procedure was employed when the light membrane fraction was further separated in a Beckman SW-27 rotor.

2.3. *Assays*

Malate synthase and other glyoxysomal enzymes were assayed as in [3]. NAD(P)H-cytochrome *c* reductase was assayed as in [10] following the A_{550} increase of reduced cytochrome *c*. Protein was precipitated with trichloroacetic acid and then estimated as in [11]. A_{280} was monitored by an ISCO UA-4 UV-monitor.

3. Results

Two malate synthase peaks, representing a soluble form and the enzyme housed in glyoxysomes, can be seen when the whole homogenate prepared from the cotyledons of day 5 cucumber seedlings was separated on a sucrose gradient by centrifugation at 100 000 × g_{av} for 3 h in a SW-27 rotor [12]. A similar procedure applied to a homogenate from cotyledons of day 3 plants affords a markedly different enzyme profile with malate synthase being also found at densities 1.09–1.12 kg/l. Similar relations were observed [13] in work with the endosperm of castor beans. It was concluded that the additional malate synthase-containing peak represents smooth endoplasmic reticulum. But if the profiles of malate synthase and antimycin A-insensitive NADH-cytochrome *c* reductase, a marker of endoplasmic reticulum, are carefully superimposed, some minor deviations can be detected.

We achieved an optimal separation of the malate synthase-containing structures by applying these conditions: high *g*-values in a zonal rotor (14-Ti) and short centrifugation periods (45 min). Figure 1 shows the results of this procedure: two peaks of endoplasmic reticulum and two malate synthase zones at densities from 1.09–1.12 kg/l. In the presence of Mg^{2+}, the position of endoplasmic reticulum could be shifted, as expected, to higher densities of approx. 1.13 kg/l.

The question whether or not the malate synthase-containing structures and smooth endoplasmic reticulum are identical was decided by prolonged centrifugation (90 min). Figure 2 gives such an example where malate synthase reached 35% sucrose with cytochrome *c* reductase remaining at 23% and 29% sucrose.

We noted that an organelle fraction buoying at 39% sucrose, containing some mitochondria and primarily membranes rich in glycoproteins, traps besides other organelles also the malate synthase-containing structures. The procedure applied in fig.2 was therefore not further pursued. A combination of sedimentation velocity centrifugation and isopycnic gradient

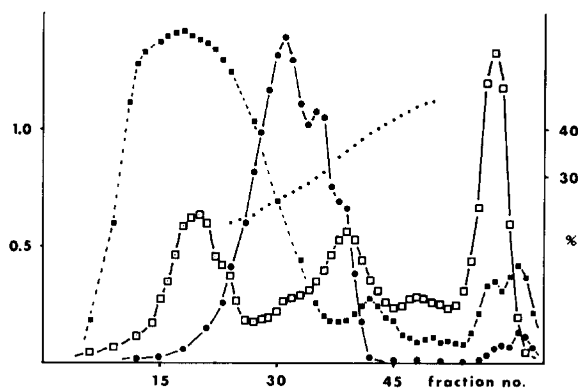

Fig.2. Prolonged zonal centrifugation of a homogenate from day 3 cotyledons. Contrary to the procedure used in fig.1, the zonal rotor spun for 90 min. Symbols and relative units (ordinate) as in fig.1.

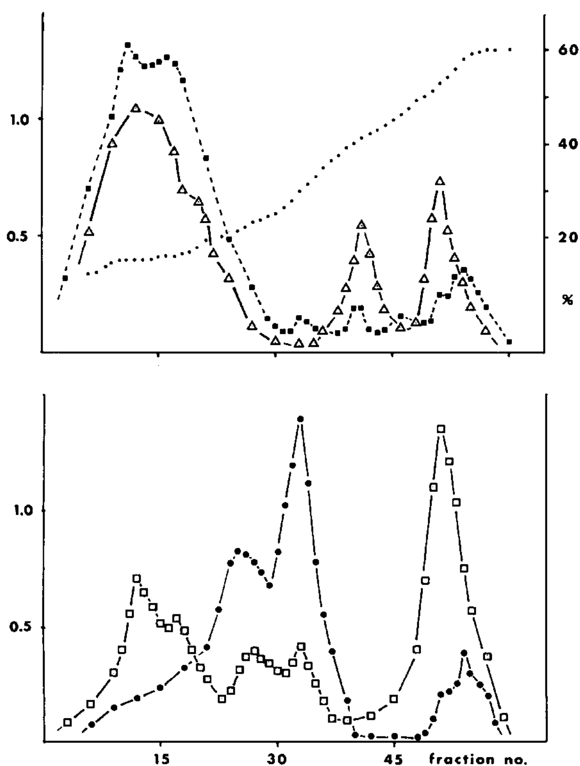

Fig.1. Zonal centrifugation of a homogenate from cotyledons of day 3 cucumber seedlings. Crude homogenate, 100 ml (equiv. 50 g cotyledons) prepared in a grinding medium containing 1 mM EDTA were layered onto a sucrose gradient (15–60%) and separated at 46 000 rev/min for 45 min in a 14-Ti zonal rotor. The ordinate represents relative units (r.u.): (–□–□–) malate synthase, 1 r.u. = 20 nkat/ml; (–•–•–) NADH-cytochrome *c* reductase, 1 r.u. = 10 nkat/ml; (–△–△–) malate dehydrogenase, 1 r.u. = 1.3 μkat/ml; (--■--■--) protein, 1 r.u. = 12 mg/ml; (···) %sucrose.

flotation turned out to be an effective means of purifying endoplasmic reticulum as well as the malate synthase-containing structures.

After the first centrifugation (fig.1), the larger organelles have almost reached their equilibrium densities while vesicles smaller than 300 S have not. Their position in the gradient is very much dependent on the centrifugation conditions used, e.g., the rotor employed. Two zones of the first gradient (see fig.1), namely with 23–26% and 27–31% sucrose, respectively, were brought to 35% sucrose, layered onto a sucrose gradient and centrifuged for 15 h. After this recentrifugation, the enzyme profiles (fig.3,4) demonstrate that the endoplasmic reticulum bands, in both cases, at 27.5% sucrose while the main proportion of malate synthase activity has reached a density of more than 1.20 kg/l (equiv. > 45% sucrose). This is still not the equilibrium density of the particles.

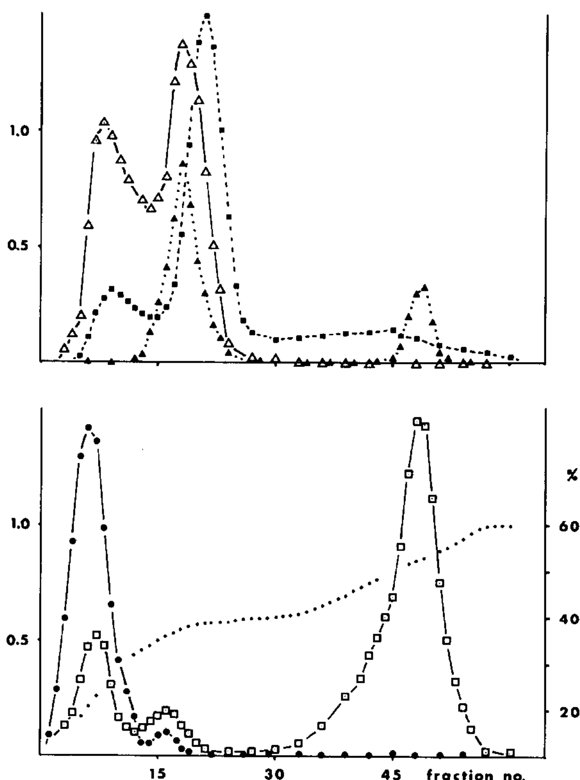

Fig.3. Recentrifugation of the heavy membrane fraction (d = 1.12 kg/l) for 15 h. The pooled fractions 30–35 from the zonal centrifugation (fig.1) were brought to 35% sucrose and layered into a sucrose gradient ranging from 10–60%. The zonal rotor 14 Ti was run at 46 000 rev/min. The ordinate represents relative units (r.u.): (–□–□–) malate synthase, 1 r.u. = 1.5 nkat/ml; (–●–●–) NADH-cytochrome c reductase, 1 r.u. = 2 nkat/ml; (–△–△–) malate dehydrogenase, 1 r.u. = 13 nkat/ml; (--■--■--) protein, 1 r.u. = 0.6 mg/ml; (–▲–▲–) citrate synthase, 1 r.u. = 1.2 nkat/ml.

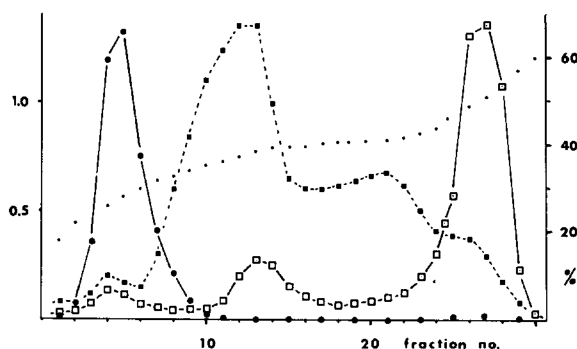

Fig.4. Recentrifugation of the light membrane fraction (d = 1.09 kg/l) for 15 h. Malate synthase peak fraction at 22% sucrose (No. 27, fig.1) 5 ml was made up to 35% sucrose and positioned within a sucrose gradient from 20–60%. The gradient was subjected to centrifugation at 27 000 rev/min for 15 h in a SW-27 rotor. Ordinate in relative units: (--■--■--) E_{280}; (–□–□–) malate synthase, 1 r.u. = 7 nkat/ml; (–●–●–) NADH-cytochrome c reductase, 1 r.u. = 1.2 nkat/ml.

As a control, it was necessary to demonstrate that the small malate synthase-containing structure is not an artifact originating from glyoxysomes. Preparations of intact glyoxysomes [3], from day 3 and day 5 seedlings as well, were shocked with buffer of low osmolarity or disintegrated by blending in a mixer for 10 s. But the disintegration of glyoxysomes did not yield a structure which behaved like the slowly sedimenting particles described in fig.3 or 4. The glyoxysomal membranes sedimented, under conditions as in fig.1, at densities between 46% and 53% sucrose, depending on the time of germination after which the cotyledons were harvested.

4. Discussion

The data obtained in the present study demonstrate that the additional malate synthase-containing cell structures which characterize the early stage of germination with most active organelle synthesis are separable from the endoplasmic reticulum. The new and

hitherto not fully characterized structures behave like small particles (< 200 S) with an unusually high equilibrium density. As they appear during the stage of glyoxysome biosynthesis and disappear when glyoxysomes are fully active, it would be reasonable to assume that they play a role in glyoxysome assembling. At the present time we cannot decide whether we are dealing with small proforms of glyoxysomes, with vesicles which have been separated from an already differentiated endoplasmic reticulum during the cell disintegration, or with portions of certain regions of endoplasmic reticulum, which are obtained artificially by the high centrifugal forces applied.

The centrifugation methods described here allow also, superior to most procedures used in plant cell fractionation, the preparation of purified endoplasmic reticulum. It is obtained by flotation centrifugation and contains a small portion of malate synthase and, in addition, traces of malate dehydrogenase (fig.3,4).

The inhomogeneity of microsomal fractions revealed here and the fact that, especially in early stages, glyoxysomes (cf. fraction 54, fig.1) and surprisingly many other fractions can be contaminated by globulins cast doubt on attempts to compare membranes of endoplasmic reticulum with those of glyoxysomes unless a thorough purification of the respective fractions preceded such investigations.

Studies to characterize the small particle malate synthase and the kinetics of its biosynthesis and turnover are in progress.

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