SELECTIVE UPTAKE BY GLYOXYSOMES OF IN VITRO TRANSLATED MALATE SYNTHASE

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1. Introduction

Malate synthase is the dominating protein of the membrane of glyoxysomes [1,2]. Studies on the biosynthesis of glyoxysomes have shown that precursor forms of malate synthase were present as a significant cytosolic pool during the assembly of the organelles [3–5]. Findings in vivo that differences in M_r between the newly synthesized cytosolic malate synthase and the glyoxysomal form of the enzyme were not detectable [4,5] argue against a co-translational transport of malate synthase into the endoplasmic reticulum and a further pathway via segregating vesicles. In contrast to [6,7] precursor forms could not be attributed to the endoplasmic reticulum [4]. Kinetic data [4] support the hypothesis that malate synthase is synthesized in the cytosol and transferred directly into the glyoxysomes.

We have assumed an import of cytosolic forms into the glyoxysomes which might also be demonstrated in vitro. These studies show that glyoxysomes contained in a mixture of several organelles and incubated with radioactive proteins, which have been newly synthesized by an in vitro system, picked up malate synthase and protected it against proteolytic enzymes.

2. Materials and methods

Cotyledons of 3-day-old cucumber seedlings (*Cucumis sativus*) were used. The growth conditions and the preparation of organelle fractions has been reported [1,4]. Poly(A) mRNA was prepared from 3-day-old cotyledons and purified on oligo(dT) cellulose (type 7, PL Biochemicals) as in [8,9]. Rabbit globin mRNA was obtained from Sigma and used as a control messenger. Enzyme assays, electrophoretic analyses and fluorography were performed as in [1,4].

An organelle fraction consisting primarily of glyoxysomes, mitochondria and proplastids was obtained from 3-day-old cotyledons as follows: Cotyledons were homogenized at 0°C by chopping with a scalpel in a grinding medium containing 15% sucrose, 150 mM Tris–HCl (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$ and 1 mM EDTA. After removing cell debris and large organelles at 2000 × *g* for 5 min, glyoxysomes and other organelles were sedimented at 10 000 × *g* for 15 min. The crude organelles were suspended in grinding medium and again centrifuged at 10 000 × *g* for 15 min. Finally the sediment was suspended in 500 μl grinding medium and supplemented with cycloheximide final concentration 0.1 mg/ml.

For in vitro translation the incubation mixture contained: 10 μl poly(A) mRNA (0.03 μg/μl) and 100 μl of a mixture of reticulocyte lysate (Amersham Buchler) and 20 μl [^{35}S]methionine (370 kBq, 30 TBq/mmol).

After incubation for 60 min, the lysate was centrifuged at 100 000 × *g* to remove the polysomes. Sucrose was added to the mixture in a final concentration of 15% and cycloheximide in a final concentration of 0.1 mg/ml).

Freshly prepared organelle fraction (200 μl) was mixed with the translation system and gently shaken for 60 min at 30°C. Subsequently, the incubation mixture was treated for 30 min at 30°C with proteinase K (final concentration 0.3 mg/ml). The reaction of proteinase K was terminated by the addition of phenylmethylsulfonylfluoride (final concentration 0.1 mM). This mixture was immediately placed onto a sucrose gradient (20–60% sucrose) and centrifuged in a SW 27 rotor (Beckman) for 90 min at 27 000 rev./min and 6°C. After the run, the gradient was fractionated into 1.2 ml fractions and aliquots of each fraction were pipetted onto a filter paper and counted. The remaining glyoxysomal fraction was diluted to obtain

a 15% sucrose concentration by addition of 50 mM Tris–HCl (pH 8) and 200 mM $MgCl_2$. This shock treatment leads to ghost formation and to the release of membranes which were sedimented at 40 000 rev./min (50 Ti rotor, Beckman) for 45 min. The supernatant was used for precipitation with anti-malate synthase antibodies as in [4].

3. Results

Direct transport into glyoxysomes of proteins formed by a soluble in vitro translation system was likely to take place since several findings excluded a co-translational transport into the endoplasmic reticulum. Neither an additional signal sequence for the in vitro product, as compared to the glyoxysomal protein, were found nor pools in the endoplasmic reticulum could be detected in in vivo experiments. Fig.1 demonstrates that, within an error of 500, the subunit M_r of the labelled malate synthase obtained from purified glyoxysomes was identical with the subunit M_r of the in vitro translated malate synthase.

Fig.2 shows the selective uptake of the labelled proteins produced in a reticulocyte in vitro translation system directed by a poly(A) mRNA from cotyledons of cucumber seedlings. By contrast, using a translation system directed by globin mRNA we did not see radioactivity at the position of glyoxysomes. Fraction 20–22 assayed by immunoprecipitation did not yield radioactive malate synthase.

Furthermore, malate synthase did not sediment in this position (fractions 21, 22) in the absence of glyoxysomes. Any 5 S newly synthesized malate synthase not destroyed by proteinase K would have been recovered in fraction 3 under these conditions.

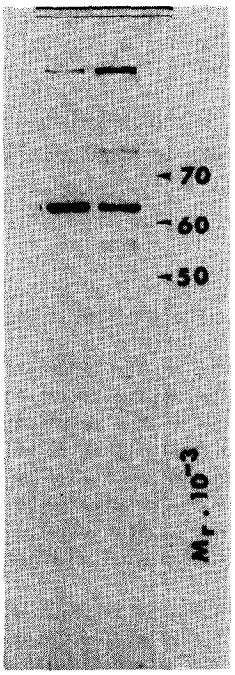

Fig.1. Comparison of subunit M_r of malate synthase purified from L-[^{35}S]methionine-labelled glyoxysomes (left) and of labelled malate synthase obtained by an in vitro translation system directed by poly(A) mRNA from cucumber cotyledons (right).

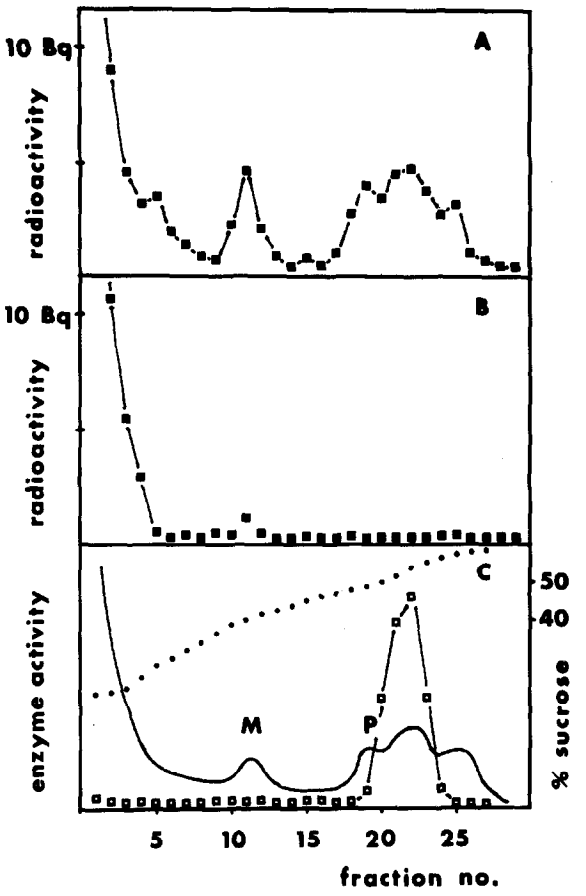

Fig.2. Separation of glyoxysomes from proplastids, mitochondria, and low M_r material by sucrose density gradient centrifugations. (A) Separation of organelles which have been incubated with the products of the in vitro translation and subsequently treated with proteinase K; (B) identical procedure, but with globin mRNA used as a messenger; (C) parallel gradient which was analysed for malate hydrolyase and cytochrome *c* oxidase (marker for mitochondria, position M), for ribulosebisphosphate carboxylase (marker for proplastids, position P) and catalase (▲——▲); (· · · ·) % sucrose; (——) E_{280}.

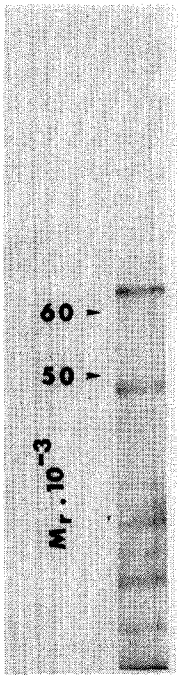

Fig.3. Electrophoretic analysis of malate synthase originating from glyoxysomes (see fig.2, fractions 21, 22) and after immunoprecipitation.

Polysomes had already been removed by centrifugation prior to incubation with the organelles.

Selectivity of uptake was observed since mitochondria and proplastides, although present during incubation and after centrifugation, lacked radioactive malate synthase.

The degree of efficiency of proteolytic degradation by proteinase K prior to centrifugation was thoroughly investigated by using different concentrations of proteinase and pursuing the fate of marker proteins. Effective proteolysis was evident as constituents of damaged protein bodies which usually contaminate the glyoxysomal fractions were absent from glyoxysomes after the proteinase K treatment. Only the molecules protected by the glyoxysomal membrane were preserved during this procedure.

Malate synthase was solubilized from the purified glyoxysomes and immunoprecipitated. The electrophoretical analysis of the precipitate (fig.3) shows mainly the M_r 63 000 band of malate synthase and a M_r 48 000 degradation product of malate synthase. The latter was frequently observed when membrane-bound malate synthase was incubated with trypsin or chymotrypsin.

4. Discussion

These results are compatible with our earlier reports on glyoxysome biosynthesis in vivo and the cytosolic precursor pools [4]. The failure of significant and preferentially labelled pools of malate synthase in the endoplasmic reticulum and the early occurrence of heavily labelled soluble malate synthase is clearly attributable to the fact that the cytosol favours a post-translational import into the organelles. The studies in [10] support a biosynthetic pathway which does not include the endoplasmic reticulum. Uptake experiments have been reported for the microbodies from *Neurospora crassa* [11].

In vitro translation and uptake can be easily separated in time. Apart from questions of how the selectivity in uptake is warranted, the molecular properties of malate synthase itself are most intriguing. Formed in the cytosol as a soluble monomer, and capable of aggregation to an octamer or a highly aggregated 100 S form, the protein later in the glyoxysomes behaves as a rather hydrophobic membrane protein. It became evident that already the cytosolic precursor form has a high affinity towards amphipathic lipids [12].

Acknowledgements

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