Lack of aconitase in glyoxysomes and peroxisomes

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The aim of this work was to find out whether aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] which is rapidly inactivated by H_2O_2, is present in the microbodies from plant cells. The separation of intact organelles from castor-bean (*Ricinus communis*) endosperm and potato (*Solanum tuberosum*) tuber indicated that aconitase activity is essentially limited to the mitochondria and cytosol fraction, but was not detected in highly purified castor-bean endosperm and potato tuber peroxisomes. An isotropic e.p.r. signal of the type expected for the 3Fe cluster of oxidized aconitase was not detected in microbodies. In immunoblot analyses, antibodies raised against potato tuber mitochondrial aconitase did not cross-react with any glyoxysomal or peroxisomal protein. Positive reactions were found for cytosol fraction and mitochondria of castor-bean endosperm. The operation of the full glyoxylate cycle in isolated glyoxysomes requires the presence of aconitase in the incubation medium. It is concluded that glyoxysomes are probably devoid of aconitase and that the glyoxylate cycle requires a detour via the cytosol, which contains a powerful aconitase activity.

INTRODUCTION

It has been clearly established that the efficient conversion of non-esterified fatty acids into sucrose in the endosperm tissue of germinating castor bean (*Ricinus communis*) depends on the channelling of acetyl-CoA produced by β-oxidation through the glyoxylate cycle [1]. The whole sequence from fatty acids to succinate is achieved within specialized peroxisomes (glyoxysomes) [2]. The five steps constituting the glyoxylate cycle are catalysed by citrate synthase (EC 4.1.3.7), aconitase (EC 4.2.1.3), isocitrate lyase (EC 4.1.3.1), malate synthase (EC 4.1.3.2) and malate dehydrogenase (EC 1.1.1.37). Except for aconitase all the enzymes of the glyoxylate cycle have been purified and investigated extensively [3]. H_2O_2 is produced continuously during the β-oxidation of fatty acids [4]. Verniquet et al. [5] reported that incubation of purified aconitase with small amounts of H_2O_2 caused the build up of an e.p.r.-detectable 3Fe cluster with a low field maximum of $g = 2.03$ leading to a progressive inhibition of aconitase activity. Consequently these results raise the problem of the presence of an aconitase in glyoxysomes or peroxisomes. Indeed considering the rapid inactivation of aconitase by small amounts of H_2O_2 and the very poor affinity of catalase for its substrate {it is not possible to saturate the enzyme with H_2O_2 within the feasible concentration range (up to 5 M H_2O_2) [6]}, it is difficult to understand how aconitase can operate in microbodies if there is a continuous production of H_2O_2. The aim of the work described here was to determine whether microbodies (peroxisome and glyoxysome) contain aconitase.

MATERIALS AND METHODS

Isolation of glyoxysomes and peroxisomes

Glyoxysomes were isolated and purified from castor bean (*Ricinus communis* L.) endosperm as described by Cooper and Beevers [7] using a step sucrose gradient. Castor beans (generously given by Prolea, Lavaur, France) were germinated 6 days in moist vermiculite. Endosperm tissue was carefully removed and the rest of the seedling, including the cotyledons, was discarded. Washed endosperm (100 g) was suspended in 150 ml of grinding medium [0.4 M sucrose/0.15 M Tricine buffer (pH 7.5)/10 mM KCl/1 mM $MgCl_2$/1 mM EDTA/2 mM dithiothreitol/0.5% (w/v) BSA/0.5% (w/v) PVP 25 (polyvinylpyrrolidone; M_r 25000; Serva Feinbiochemica, Heidelberg, Germany)] and chopped with razor blades in order to avoid the rupture of the fragile glyoxysomes. After filtration through two layers of cheesecloth the crude total extract was centrifuged for 10 min at 280 *g* (Sorvall SS 34 rotor) to remove unbroken cells and cellular debris. The supernatant solution was re-centrifuged for 30 min at 10800 *g* (Sorvall SS 34 rotor), yielding the supernatant and the crude particulate pellet. The pellet was then suspended in 2–5 ml of grinding medium, and 2 ml of the resulting suspension was layered on a step sucrose gradient containing 10 mM Tricine buffer, pH 7.5, and 1 mM EDTA. The step gradient, consisting of 2 ml of 60% (w/w) sucrose, 7 ml of 57% (w/w), 10 ml of 50% (w/w), 10 ml of 44% (w/w) and 7 ml of 33% (w/w) was used immediately after preparation. The tubes were centrifuged at 120000 *g* (SW 27 swinging-bucket rotor; Sorvall OTD2 ultracentrifuge) for 5 h at 4 °C. At the conclusion of this step glyoxysomes and mitochondria are clearly separated [7]. Purified glyoxysomes and mitochondria were collected from the gradient by aspiration.

Peroxisomes were isolated from potato (*Solanum tuberosum* L.) tubers as described by Neuburger et al. [8] using self-generating Percoll (Pharmacia) gradients. The peroxisomes were recovered as a sharp dark band sitting above a clear colourless layer of Percoll at the bottom of the tube, and intact mitochondria were recovered as a broad band located beneath a yellow layer at the top of the gradient [8]. The peroxisomal fraction was collected with a Pasteur pipette. This fraction was diluted 10-fold in the washing medium [10 mM KH_2PO_4 (pH 7.2)/0.3 M mannitol/1 mM EDTA] [8]. The peroxisomes were then sedimented at 5000 *g* for 15 min. Unfortunately, even after centrifugation by isopycnic centrifugation in density gradients of Percoll, peroxisomes are still contaminated with mitochondria. Thus the following purification procedure is repeated twice, that is, after pelleting, peroxisomes were layered at the top of a new 50% Percoll medium, leading to a pure and intact preparation of

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Table 1 Subcellular localization of pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PFP; cytosolic marker), malate synthase (glyoxysomal marker), fumarase (mitochondrial marker) and aconitase in mechanically ruptured castor-bean endosperm, followed by centrifugation (10800 *g* for 30 min)

Preparation of chopped endosperm (100 g) and centrifugation of intact organelles were carried out as described in the Materials and methods section. Enzymic activities were measured in 0.5 ml of the appropriate reaction medium (see the Materials and methods section) using spectrophotometric methods. Values in the Table are expressed for 100 g of endosperm in μmol of *cis*-aconitate formed/min for aconitase ($\epsilon_{240} = 4880\ M^{-1} \cdot cm^{-1}$); μmol of NADH consumed/min for pyrophosphate:fructose-6-phosphate 1-phosphotransferase (ϵ_{340} 6230 $M^{-1} \cdot cm^{-1}$); μmol of 5,5′-dithiobis-(2-nitrobenzoic acid) consumed/min for malate synthase (ϵ_{412} 13600 $M^{-1} \cdot cm^{-1}$); μmol of fumarate formed/min for fumarase (ϵ_{240} 2400 $M^{-1} \cdot cm^{-1}$). The data are from a representative experiment that was reproduced three times. Since tissue disruption was carried out very gently, the maximum yield of intact organelles was very low (less than 5% of the total tissue cell organelles).

	Enzyme activity (μmol/min)			
Fraction	Aconitase	PFP	Malate synthase	Fumarase
Supernatant	13	8.3	3.5	0.2
Pellet	1.2	0.5	14	35

Table 2 Measurement of various markers and aconitase in purified cell organelles (mitochondria and glyoxysomes) isolated from castor-bean endosperm

The separate preparations of cell organelles are described in the text. Enzymic activities were measured in 0.5 ml of the appropriate reaction medium (see the Materials and methods section) using spectrophotometric methods. Values in the Table are expressed in nmol of *cis*-aconitate formed/min per mg of protein for aconitase (ϵ_{240} 4880 $M^{-1} \cdot cm^{-1}$); nmol of 5,5′-dithiobis-(2-nitrobenzoic acid) consumed/min per mg of protein for malate synthase (ϵ_{412} 13600 $M^{-1} \cdot cm^{-1}$); nmol of fumarate formed/min per mg of protein for fumarase ($\epsilon_{240} = 2400\ M^{-1} \cdot cm^{-1}$). The data are from a representative experiment that was performed three times.

	Specific activity (nmol/min per mg of protein)		
Fraction	Aconitase	Malate synthase	Fumarase
Matrix of isolated mitochondria	48	25	961
Isolated glyoxysomes	0	714	0

peroxisomes. The black pellet thus obtained was stored at approx. 20 mg of protein/ml under argon to protect aconitase against oxidation.

Isolation of mitochondria and purification of aconitase

Mitochondria were isolated and purified from potato tubers as described by Neuburger et al. [8] by using a self-generating Percoll gradient. The mitochondria were more than 95% intact as judged by their impermeability to cytochrome *c* [8]. Total release of the matrix protein was achieved by three cycles of freezing and thawing as described by Neuburger et al. [9]. The suspension of broken mitochondria was centrifuged at 100000 *g* for 2 h (35000 rev./min in a Sorvall A6.41 rotor) to remove all the mitochondrial membranes. The supernatant thus obtained (120 ml; 4 mg of protein/ml) was filtered through a 0.22 μm-pore-size filter (Sterivex-GS, Millipore). Aconitase was purified from the matrix extract as described by Verniquet et al. [5].

Immunochemical studies of aconitase

Purified aconitase was checked for purity in SDS/7.5–15% (w/v) polyacrylamide-gradient gels. Bands corresponding to aconitase were excised and pooled. The portions of gel were chopped and injected as an emulsion with Freund's adjuvant into rabbits for raising antibodies. Booster injections (500 μg of native enzyme) were given at 20 day intervals. Blood was collected 10 days after the third injection. IgG fractions were purified from rabbit antisera by ion-exchange chromatography as described by Saint-Blancard et al. [10]. Pooled fractions containing IgG were dialysed and concentrated by ultrafiltration (XM-50 membrane; Amicon). Purified IgG fractions (0.01 ml; 50 mg/ml in 10 mM Tris/HCl, pH 8.3) were stored at −80 °C until used.

Electrophoretic analyses of soluble proteins

Soluble proteins were analysed by SDS/PAGE, which was performed at room temperature in slab gels containing a 7.5–15%-acrylamide gradient [10]. Polypeptides from the different fractions analysed were also transferred electrophoretically to nitrocellulose (Bio-Rad) sheets essentially as described by Towbin et al. [11], using a Trans-Blot Cell semi-dry system (Bio-Rad). The electrophoresis was performed at room temperature in 25 mM Tris/HCl/192 mM glycine/15% (v/v) propan-2-ol/0.01% (w/v) SDS; the power range was 20 V at 3 mA/cm^2. The transfer was complete after about 30 min. The sheets were then stained with Ponceau S for detection of soluble peptides that were transferred.

Immunoblotting studies

We used polyclonal antibodies raised against purified aconitase from potato tuber mitochondria. Immunoblotting experiments were performed essentially as described by Burnette [12]. Antibody binding was determined by using alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) and staining for alkaline phosphatase activity.

Measurement of enzyme activities

Cell organelles were diluted in lysis buffer [50 mM Mops (pH 7.5)/5 mM $MgCl_2$]. Total release of the matrix protein was achieved by three cycles of freezing and thawing. The suspension of broken organelles was centrifuged at 150000 *g* for 20 min [Beckman Airfuge; 862.5 kPa (125 lbf/in^2)] to remove all the membrane systems. Activities were measured in the supernatant. All assays were optimized with respect to the concentration of each component and to the pH of the reaction mixture. For the reactions that were monitored with a spectrophotometer (Kontron Uvikon 810), the coupling enzyme systems were checked not to be rate-limiting. References to the procedures are given together with any features of the reaction mixtures that differed from those in the references. Aconitase (EC 4.2.1.3) activity was assayed by monitoring the appearance of *cis*-aconitate at 240 nm as a function of time (ϵ_{240} 4880 $M^{-1} \cdot cm^{-1}$) [13]. Pyrophosphate:fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90) activity was measured as the pyrophosphate-dependent conversion to fructose 6-phosphate into fructose 1,6-bisphosphate [14]. The assay mixture contained 50 mM Hepes/NaOH, pH 7.8, 0.5 mM $MgCl_2$, 10 μM fructose 2,6-bisphosphate, 5 mM fructose 6-phosphate, 10 units of triose-

Table 3 Measurement of various markers and aconitase in purified cell organelles (mitochondria and peroxisomes) isolated from potato tuber

The separate preparations of cell organlles are described in the text. Enzymic activities were measured in 0.5 ml of the appropriate reaction medium (see the Materials and methods section), using spectophotometric methods. Values in the Table are expressed in nmol of *cis*-aconitate formed/min per mg of protein for aconitase (ϵ_{240} 4880 $M^{-1} \cdot cm^{-1}$); nmol of NADH formed/min per mg of protein for 3-hydroxyacyl-CoA dehydrogenase (ϵ_{340} 6230 $M^{-1} \cdot cm^{-1}$); nmol of fumarate formed/min per mg of protein for fumarase (ϵ_{240} 2440 $M^{-1} \cdot cm^{-1}$). The data are from a representative experiment that was performed three times.

	Specific activity (nmol/min per mg of protein)		
Fraction	Aconitase	3-Hydroxyacyl-CoA dehydrogenase	Fumarase
Matrix of isolated mitochondria	227	9	864
Isolated peroxisomes	0	130	0

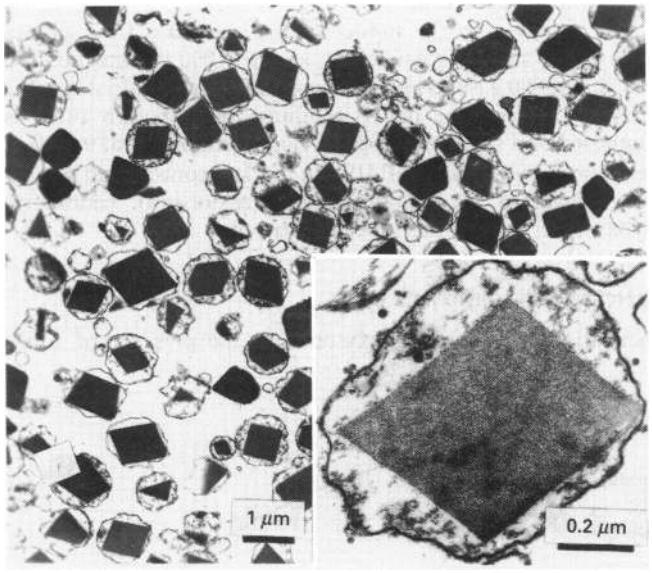


Figure 1 Electron micrographs of Percoll purified potato tuber microbodies

Fixation methods were described by Neuburger et al. (1982) [8]. Note that isolated purified peroxisomes are surrounded by a single membrane; they show no contamination with intact mitochondria.

phosphate isomerase (EC 5.3.1.1), 1 unit of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 0.1 unit of aldolase (EC 44.1.2.13), 0.15 mM NADH and 0.6 mM $Na_4P_2O_7$ (ϵ_{340} 6230 $M^{-1} \cdot cm^{-1}$). Fumarase (EC 4.2.1.2) (ϵ_{240} 2440 $M^{-1} \cdot cm^{-1}$) [15], malate synthase (EC 4.1.3.2) (ϵ_{412} 13600 $M^{-1} \cdot cm^{-1}$) [7] and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (ϵ_{412} 13600 $M^{-1} \cdot cm^{-1}$) [16] were assayed by the methods given in the cited references.

E.p.r. measurements

These were recorded on a Varian E 109 spectrometer coupled to a Hewlett–Packard 9826 computer. The samples were cooled with a liquid-helium transfer system (Oxford Instruments; ESR900) for variable temperature down to 4.2 K. Temperature was monitored with a gold–iron/chromel thermocouple about 2 cm below the bottom of the e.p.r. sample in the flowing helium-gas stream. The magnetic field was calibrated using a Varian gaussmeter. Samples of glyoxysomes or mitochondria were placed in e.p.r. quartz tubes, frozen rapidly in liquid N_2 and stored at 77 K until used.

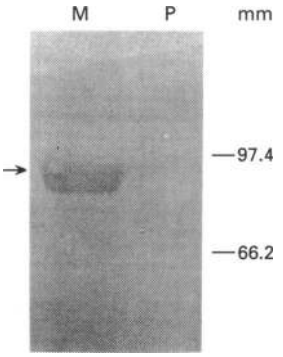


Figure 2 Western-blot analysis of mitochondrial and peroxisomal proteins with antibodies raised against potato tuber mitochondrial aconitase

Mitochondrial and peroxisomal polypeptides from potato tuber were resolved by SDS/PAGE, transferred to nitrocellulose sheet and screened with rabbit antibodies raised against potato tuber mitochondrial aconitase (see the Materials and methods section). Track M, mitochondrial matrix proteins (50 μg); track P, peroxisomal proteins (250 μg); Track mm, M_r markers (Bio-Rad laboratories). The arrow indicates the position of mitochondrial aconitase stained with Ponceau S.

RESULTS

Distribution of aconitase

This was investigated in castor-bean endosperm and potato tubers. The following marker enzymes were used: mitochondria, fumarase; peroxisomes, 3-hydroxyacyl-CoA dehydrogenase; cytosol, pyrophosphate:fructose-6-phosphate 1-phosphotransferase; glyoxysomes, malate synthase. The gentle grinding of castor-bean endosperms with razor blades followed by centrifugation carried out in two steps (see the Materials and methods section) produced a supernatant that contained an appreciable proportion of the aconitase activity. Indeed, more than 90% of the total amount of aconitase activity present in the crude homogenate was recovered in the supernatant. This was not due to mitochondrial and glyoxysomal contamination of the supernatant, since no more than 1% of the fumarase and 20% of the malate synthase were in the supernatant. The presence of an appreciable malate synthase activity in the supernatant as previously shown by Cooper and Beevers [7] is probably due to the rupture of the fragile membrane surrounding the glyoxysomes during the grinding of the tissue. The observation that almost all of the pyrophosphate:fructose 6-phosphate 1-phosphotransferase was in the supernatant is consistent with the location of at least some of the aconitase in the cytosol (Table 1).

With the aim to localize further aconitase in the crude particulate pellet containing cell organelles, intact glyoxysomes and mitochondria were isolated from castor-bean endosperm by the method of Cooper and Beevers [7]. Assays of various markers (pyrophosphate:fructose-6-phosphate 1-phosphotransferase, malate synthase and fumarase) (Tables 1 and 2) showed that centrifugation of glyoxysomes through a discontinuous sucrose gradient almost completely eliminates cytosolic and mitochondrial contamination. Table 2 clearly indicates that aconitase activity was not associated with castor-bean endosperm glyoxysomes. This unexpected result could be attributable to a pro-

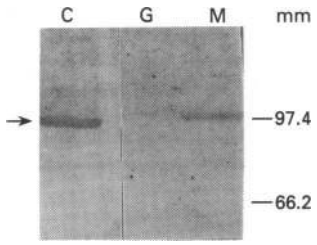


Figure 3 Western-blot analysis of glyoxysomal, mitochondrial and cytosolic fractions of castor-bean endosperm with antibodies raised against potato tuber mitochondrial aconitase

Glyoxysomal, mitochondrial and cytosolic castor-bean polypeptides were resolved by SDS/PAGE, transferred to nitrocellulose sheet and screened with antibodies raised against potato tuber mitochondrial aconitase (see the Materials and methods section). Preparation of cytosolic, mitochondrial and glyoxysomal extract were carried out as described in the Materials and methods section. Track C, cytosolic proteins (250 μg); track G, glyoxysomal proteins (250 μg); track M, mitochondrial proteins (250 μg). The arrow indicates the position of mitochondrial aconitase stained with Ponceau S.

gressive inactivation of aconitase during the course of glyoxysome isolation. Indeed very often during oxidation or purification of aconitase one Fe atom is lost, leaving a e.p.r.-detectable $[3Fe\text{-}4S]^+$ cluster associated with inactivation of the enzyme [17]. However, this hypothesis is most unlikely, because attempts to re-activate glyoxysomal aconitase by anaerobic incubation for 30 min at room temperature with an excess of Fe^{2+} and a thiol such as cysteine or dithioerythritol were unsuccessful. Furthermore, the e.p.r. spectrum of the soluble fraction of H_2O_2-treated mitochondria revealed the presence of a nearly isotropic signal with a low field maximum at $g = 2.03$, which identifies the 3Fe cluster of inactive aconitase. By contrast this signal was not detectable in glyoxysomes, even after oxidation by H_2O_2 (results not shown). Interestingly Table 3 also indicates that intact peroxisomes (Figure 1) isolated from potato tubers, in contrast with mitochondria, were devoid of aconitase activity (see also [19]). To provide further evidence for this conclusion, we used an antibody to mitochondrial aconitase to recognize any protein in glyoxysomes and peroxisomes.

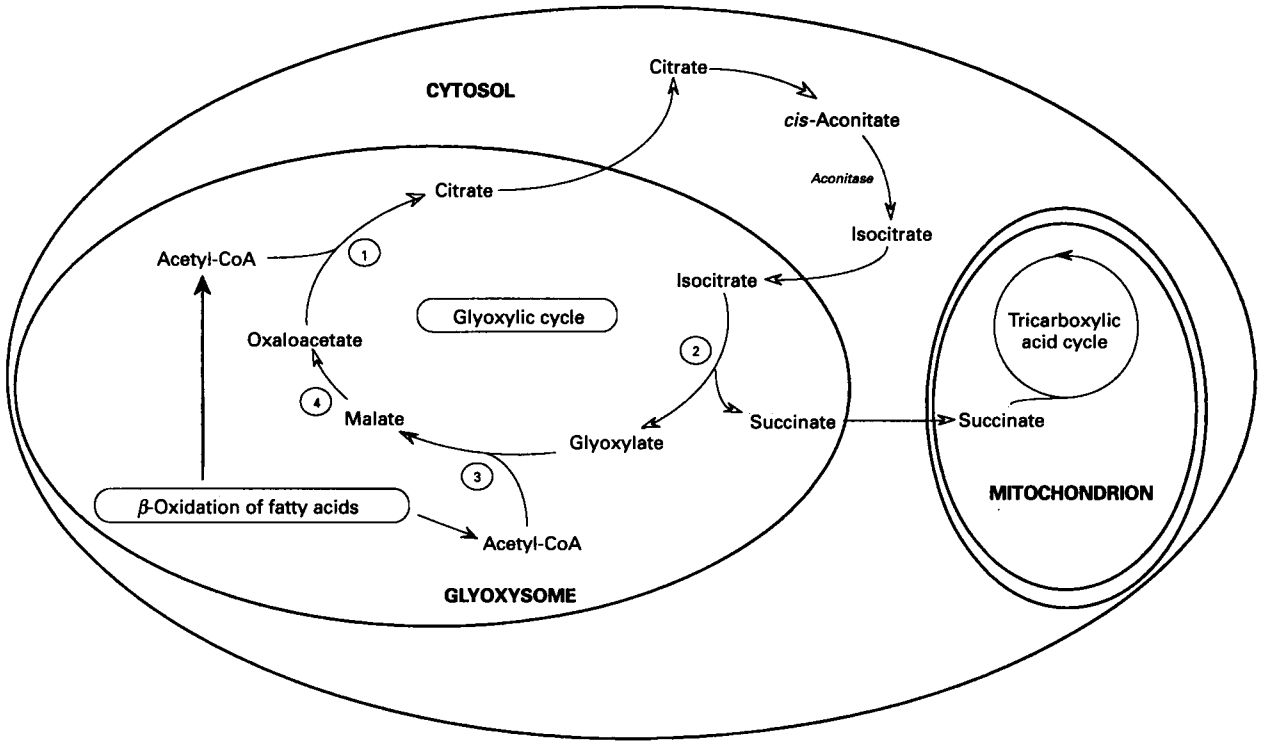
Western-blot analysis

Mitochondria, glyoxysomes and soluble proteins (10800 *g* supernatant; see the Materials and methods section) from castor-bean endosperm as well as mitochondria and peroxisome proteins from potato tuber were subjected to SDS/PAGE. The separated proteins were then transferred to nitrocellulose sheets and aconitase was detected by immunodetection with antibody against potato tuber mitochondrial aconitase. The results are shown in Figures 2–3; each experiment was repeated three times. Different samples were used on each occasion, and similar results were obtained each time.

The antibody to the mitochondrial aconitase from potato tubers recognized proteins of M_r about 94000 in mitochondria and the 10800 *g* supernatant devoid of mitochondrial marker activities. Indeed the native aconitase from potato tuber is composed of a single polypeptide chain with an M_r of 94000 as determined by SDS/PAGE [19]. In marked contrast, no protein was recognized by the antibody in castor-bean endosperm glyoxysomes and potato tuber peroxisomes.

Operation of the glyoxylate cycle

Incubation of purified glyoxysomes in the presence of 1 mM



Scheme 1 Compartmentation of the enzymes involved in the glyoxylic pathway

(1), Citrate synthase; (2), isocitrate lyase; (3), malate synthase; (4), malate dehydrogenase.

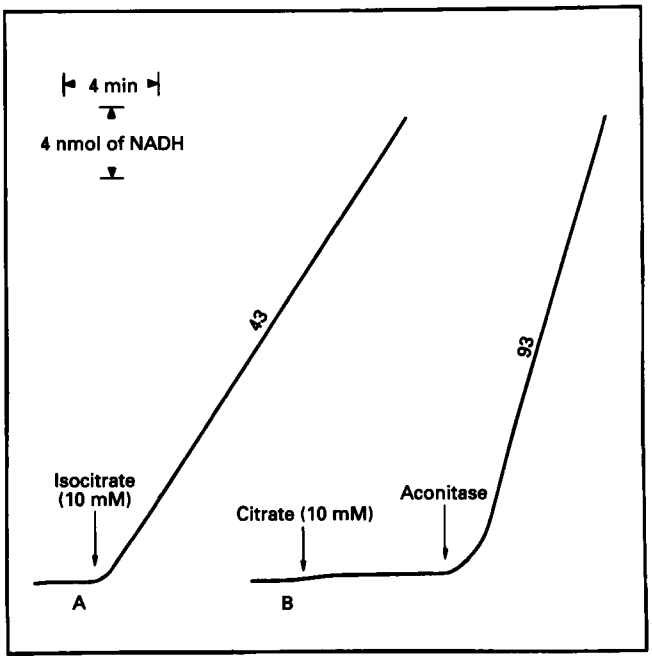


Figure 4 NAD^+ reduction by isolated glyoxysomes in the presence of isocitrate (trace A) or citrate (trace B)

The preparation of isolated glyoxysomes from castor-bean endosperm is described in the Materials and methods section. The standard essay mixture contained 0.1 M Hepes/NaOH, pH 8.0, 5 mM $MgCl_2$, 2 mM NAD^+, 1 mM acetyl-CoA and 56 μg of glyoxysomal proteins in a final volume of 0.5 ml. Reduction of NAD^+ was measured by monitoring the formation of NADH at 340 nm with a Kontron Uvikon 810 spectrophotometer. Activity was initiated either with 10 mM isocitrate (trace A) or 10 mM citrate + 190 ng of aconitase (trace B). The values on the traces refer to nmol of NADH formed/min per mg of glyoxysomal protein. Note that, in the presence of citrate, NAD^+ reduction occurs only if exogenous potato tuber mitochondria aconitase is present in the assay medium. Note also that the rate of NAD^+ reduction in the presence of 10 mM isocitrate is lower than that in the presence of 10 mM citrate + aconitase. In fact we have verified that citrate lyase (the last enzyme involved in the linear reduction of NAD^+) is inhibited by an excess of isocitrate.

acetyl-CoA, 2 mM NAD^+ and 10 mM isocitrate (glyoxysomes purified in sucrose gradients were permeable to low-M_r compounds [18]) led to an acetyl-CoA-dependent NAD^+ reduction (Scheme 1 and Figure 4, trace A). The requirement for acetyl-CoA strongly suggested that isocitrate lyase and malate synthase were producing malate which could then be used as substrate for malate dehydrogenase. In marked contrast, when the same experiment is repeated in the presence of citrate instead of isocitrate, the reduction of NAD^+ did not occur (Figure 4, trace B). However, under these conditions the addition of potato tuber mitochondrial aconitase to the incubation medium resulted in resumption of NAD^+ reduction (Figure 4, trace B). Again these results strongly suggest that aconitase was not present in the glyoxysomes and that exogenous aconitase was required for the operation of the glyoxylate cycle.

DISCUSSION

The separation of the intact organelles from castor-bean endosperm gently chopped with razor blades clearly indicates that aconitase activity is essentially limited to the mitochondria (about 10%) and cytosol fractions (90%). A recent report from our laboratory [19] also showed that sycamore (*Acer pseudoplatanus*) cells contain two isoenzymes of aconitase which are localized in different intracellular compartments, namely the mitochondria and the cytosol. Interestingly Cooper and Beevers [7] in their original publication on enzyme constituents of mitochondria and glyoxysomes from castor-bean endosperm reported that, although the total amount of aconitase present in the crude homogenate was relatively high, less than 10% of this was recovered in the crude particulate fraction, indicating that the bulk of aconitase activity was in fact localized in the cytosolic compartment.

Collectively, all the experimental approaches that we have used, including Western blotting of glyoxysomal and peroxisomal extracts using antibody to mitochondrial aconitase, e.p.r. measurements and biochemical experiments, agree, and the results lead us to conclude that intact microbodies isolated from castor-bean endosperm and potato tuber lack significant aconitase activities. Indeed such a result is very surprising, because it is generally believed that the glyoxysomal aconitase is part of the glyoxylate cycle which converts citrate into isocitrate and that the most likely organic acids to be excreted from the glyoxysome are succinate and malate, which are converted into oxaloacetate and then into phosphoenolpyruvate for glycolysis. Our results would also require, therefore, export of citrate from the glyoxysome, probably by free diffusion [20], its cytosolic conversion into isocitrate, and transport of isocitrate into the glyoxysome for use by isocitrate lyase, one of the key enzymes of the glyoxylate cycle (Scheme 1) [21]. In other words, probably because aconitase is a very fragile enzyme which is rapidly inactivated by H_2O_2 [5], the glyoxylate cycle requires a detour via the cytosol, which contains a powerful aconitase activity [19].

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