Purification and Comparative Properties of Microsomal and Glyoxysomal Malate Synthase from Castor Bean Endosperm¹,²

LINDA BOWDEN AND J. MICHAEL LORD
Postgraduate School of Biological Sciences, University of Bradford, Yorkshire, BD7 1DP, England

ABSTRACT
Sucrose density gradient centrifugation was employed to separate microsomes, mitochondria, and glyoxysomes from homogenates prepared from castor bean (Ricinus communis) endosperm. In the case of tissue removed from young seedlings, a significant proportion of the characteristic glyoxysomal enzyme malate synthase was recovered in the microsomal fraction. Malate synthase was purified from both isolated microsomes and glyoxysomes by a procedure involving osmotic shock, KCl solubilization, and sucrose density gradient centrifugation. All physical and catalytic properties examined were identical for the enzyme isolated from both organelle fractions. These properties include a molecular weight of 575,000, with a single subunit type of molecular weight 64,000, a pH optimum of 8, apparent Kₐ for acetyl-CoA of 10 μM and glyoxylate of 2 μM. Microsomal and glyoxysomal malate synthases showed identical responses to various inhibitors. Adenine nucleotides were competitive inhibitors with respect to acetyl-CoA, and oxalate (Kₐ 110 μM) and glycolate (Kₐ 150 μM) were competitive inhibitors with respect to glyoxylate. Antiserum raised in rabbits against purified glyoxysomal malate synthase was used to confirm serological identity between the microsomal and glyoxysomal enzymes, and was capable of specifically precipitating ³⁵S-labeled malate synthase from KCl extracts of both microsomes and glyoxysomes isolated from castor bean endosperm tissue.

Ultrastuctural observations of microbody-containing cells have frequently shown these organelles adjacent to sections of RER. Such observations have been interpreted as indicative of an ontogenetic relationship between these cellular fractions. The widely accepted model for this relationship is that microbodies arise from the ER by a process of vesiculation, the characteristic proteins of the microbody matrix having been synthesized on bound ribosomes, segregated by the ER membrane, and temporarily housed in the ER cisternae (16). Biochemical studies to date have provided only partial support for this model, the strongest evidence indicating a developmental relationship between the ER and microbody membranes. In particular, studies into the origin of microbodies (glyoxysomes) in germinating fatty seedlings have established that the ER and glyoxysomal membranes are similar in their phospholipid (12, 13) and polypeptide (4) composition, that the major phospholipid components of the glyoxysomal membrane are synthesized by enzymes exclusively located in the ER (3, 20, 23), and that the kinetics data of both phospholipid (18) and protein (5) labeling from radioactive precursors support a direction of membrane flow from the ER to the glyoxysome.

Attempts to demonstrate that enzymic proteins of the microbody matrix are initially segregated by the ER, particularly in the case of peroxisomal catalase of rat liver, have failed to provide evidence consistent with the model described above. These studies have been interpreted as indicating that newly synthesized catalase is released into the cytosol before rapidly appearing in the peroxisomes (19). A more recent study by Gonzalez and Beevers (14) has shown that a significant proportion of the activity of the glyoxysomal enzymes malate synthase and citrate synthase is associated with the microsomal fraction during the early postgerminative growth of castor bean seedlings, a time when rapid glyoxysome formation is occurring (1). This finding is clearly consistent with the current model for microbody biogenesis. Before it can be taken as valid evidence for this model it is necessary to show that microsomal and glyoxysomal malate synthases are identical and that the activity present in the microsomal fraction is ultimately transported to the glyoxysomes. In the present paper microsomal and glyoxysomal malate synthase proteins isolated from the endosperm of young castor bean seedlings are shown to be identical in their physical, catalytic, and serological properties examined.

MATERIALS AND METHODS
Plant Material. Seeds of castor bean (Ricinus communis) were soaked overnight in running tap water and were grown in moist vermiculite at 30 C. After the appropriate period of germination the seedlings were detached at the hypocotyl, the endosperm tissue was removed, and the cotyledons were discarded. Cellular proteins were radioactively labeled by incubating the intact endosperm tissue with [³⁵S]methionine. Endosperm halves were placed abaxial surface down on moist filter paper and 10 μCi of L-[³⁵S]methionine (465 Ci/mmol; Radiochemical Centre, Amersham, Bucks., U.K.) was applied to the inner surface of each endosperm half. The tissue was incubated for 3 hr at 30 C in darkness in covered dishes.

Separation of Cellular Organelles. Homogenization of endosperm tissue and the separation of organelles by sucrose density gradient centrifugation were performed exactly as described previously (4). In order to increase the yield of cellular organelles recovered from gradients in enzyme purification experiments, crude homogenates were centrifuged at 20,000g for 10 min. The organelle pellets thus sedimented were gently resuspended in 5 ml of grinding medium prior to gradient centrifugation, allowing the organelles isolated from 20 endosperm halves to be added to each gradient. After centrifugation, 1-ml fractions were collected by using a Beckman gradient fractionator. For the large scale purification of glyoxysomes used to purify larger quantities of malate...
synthase for antibody production, crude homogenates were fractionated on linear 30 to 60% (w/w) sucrose gradients constructed in a 1,600-ml volume B vial X zonal rotor and centrifuged for 6 hr at 20,000 rpm on an MSE "Superspeed 40" centrifuge. In this way, 50 to 60 g of endosperm tissue could be fractionated.

**Purification of Malate Synthase.** Sucrose gradient fractions which contained the protein bands recovered at mean buoyant densities of 1.12 and 1.24 g/ml (that is, the microsomes and glyoxysomes, respectively) were separately pooled. An equivalent volume of 50 mM Tricine (pH 7.5) was added to each pooled sample to disrupt the organelles osmotically (17). After shaking in a Vortex mixer, these suspensions were incubated at 25 C for 30 min. The malate synthase-containing membrane vesicles were recovered by centrifuging at 100,000g and 2 C for 30 min. Membrane pellets were resuspended in 1 ml of 0.2 M KCl and the resulting suspensions were subjected to three 30-sec bursts of ultrasonication on an MSE ultrasonicator operating at maximum output. The sonicated membrane suspensions were incubated at 5 C overnight. The KCl-solubilized material was separated from the membranes by centrifuging at 100,000g and 2 C for 30 min. The malate synthase-containing supernatants were layered onto gradients which consisted of 32 ml of sucrose solution increasing linearly in concentration from 5 to 25% (w/w) and contained in 38.5-mL cellulose nitrate tubes. Sucrose solutions contained 1 mM MgCl2 and 0.2 M KCl. Gradients were centrifuged at 24,000 rpm (80,000g average) and 2 C for 15 hr in a SW 27 rotor in a Beckman L5 40 ultracentrifuge. After centrifugation, 1-ml fractions were collected and assayed for malate synthase activity.

**Analytical Sucrose Gradient Centrifugation.** The mol wt of purified malate synthase was estimated by comparing its rate of sedimentation in 32-mL linear 5 to 25% (w/w) sucrose gradients with the rates of sedimentation of proteins of known mol wt. Each gradient was overlaid with a 2-ml sample containing either 0.25 mg of purified malate synthase or 1 mg of the following reference proteins: pig heart NADP-linked isocitrate dehydrogenase (mol wt 60,000), beef liver glutamate dehydrogenase (mol wt 320,000), Jack bean urease (mol wt 480,000), and Escherichia coli \( \beta \)-galactosidase (mol wt 540,000). Gradients were simultaneously centrifuged at 24,000 rpm (80,000g average) and 2 C for 15 hr. Fractions (1 ml average) were collected and protein peaks were located by measuring A at 280 nm, and the presence of active enzyme confirmed by direct enzymic assay.

**Gel Filtration.** The mol wt of malate synthase was also estimated by gel filtration at 2 C on a Sepharose 6B column (2 x 90 cm). Approximately 0.25 mg of malate synthase was applied to the column which had been equilibrated with 50 mM Tris (pH 8) containing 0.2 M KCl and 1 mM MgCl2; 3-ml fractions were collected. The column was calibrated with the following standards: urease (mol wt 480,000), \( \beta \)-galactosidase (mol wt 540,000), and Euglena gracilis ribulose 1,5-diP carboxylase (mol wt 550,000). Elution profiles were established by measuring enzymic activity (malate synthase) or [3H]methionine (standard) of collected fractions.

**Polyacrylamide Gel Electrophoresis.** Purified malate synthase was subjected to electrophoresis on disc gels polymerized from 5% (w/v) acrylamide, with a N,N'-methylene bisacrylamide to acrylamide ratio of 1:50. The gels (each loaded with approximately 10 \( \mu \)g of protein) were run for 1 hr at a constant current of 8 mamp/tube with 1% Tris in 25 mM Na-phosphate (pH 7.6) as the electrophoresis buffer and bromophenol blue as the tracker dye. Gels were either stained in Coomassie blue, destained and scanned in a Gilford gel scanner at 600 nm, or incubated in the malate synthase assay reaction mixture and immediately scanned at 412 nm.

For quaternary structure studies, malate synthase was incubated overnight at 37 C in 10 mM Na-phosphate (pH 7.5) containing 1% (w/v) SDS and 1% (v/v) \( \beta \)-mercaptoethanol and examined by electrophoresis in buffers containing SDS as described by Weber and Osborn (27) on gels polymerized from 10% (w/v) acrylamide. Soybean trypsin inhibitor was used as a molecular weight marker, and purified E. coli RNA polymerase (subunit mol wt 39,000, 155,000, and 165,000) were used to calibrate the gels.

**Preparation of Antiserum to Glyoxysomal Malate Synthase.** Purified glyoxysomal malate synthase (1 mg) in 0.5 ml was emulsified with an equivalent volume of Difco complete Freund's adjuvant and injected intramuscularly into the hind leg of a rabbit. After 2 weeks, the rabbit received a booster injection providing the same amount of antigen. After a further 2 weeks, blood was withdrawn by cardiac puncture. Null serum was obtained in identical manner from a control rabbit. Whole antiserum prepared from these blood samples was used in enzyme inhibition, immunoprecipitation, and gel diffusion analyses.

**Gel Diffusion Analysis.** Ouchterlony plates were prepared with 0.8% (w/v) agarose in 0.1 M K-phosphate buffer (pH 7.5) containing 0.01% (w/v) sodium azide as preservative. Diffusion was allowed to proceed for 36 to 48 hr at ambient temperature. Gels were then flooded with 1.7% (w/v) saline solution for 24 hr and washed over a further 24 hr with several changes of distilled H2O. Gels were stained in Coomassie brilliant blue for 2 hr and destained in 0.5% (v/v) methanol-0.75% (v/v) acetic acid solution.

**Immunoprecipitation.** Malate synthase preparations were incubated with an equivalent volume of either antiglyoxysomal malate synthase serum or null serum for 1 hr at 37 C and overnight at 2 C. Immunoprecipitates formed were recovered by centrifugation and washed three times with 0.1 M Tris (pH 7.5) containing 50 mM KCl, 10 mM MgCl2, 5 mM \( \beta \)-mercaptoethanol, and 0.5% (v/v) Triton X-100, and once in the same solution from which Triton X-100 had been omitted. Washed immunoprecipitates were redissolved in 100 \( \mu \)l of 10 mM Na-phosphate (pH 7.5) containing 1% (v/v) SDS and 1% (v/v) \( \beta \)-mercaptoethanol, and incubated overnight at 37 C prior to SDS-polyacrylamide gel electrophoresis carried out exactly as described above. Gels obtained for \[^{35}S\]methionine-labeled malate synthase were frozen on powdered solid CO2 and sliced into 1-mm sections by using a Mickel gel slicer. Each gel slice was solubilized in 0.2 ml of H2O2 (30 volumes) for 2 to 3 hr at 70 C and mixed with 10 ml of Bray's (6) scintillation fluid. Radioactivity was determined by liquid scintillation counting at 60% counting efficiency.

**Inhibition of Malate Synthase Activity by Antiserum.** Varying volumes of antiglyoxysomal malate synthase serum (up to 100 \( \mu \)l) were added to 5 \( \mu \)g of purified microsomal or glyoxysomal malate synthase and the final volume of each mixture was adjusted to 200 \( \mu \)l. After 30 min incubation at 25 C, aliquots of each mixture were removed and assayed for malate synthase activity.

**Enzyme Assays.** Malate synthase (EC 4.1.3.2) was assayed by the method of Hock and Beevers (15). The reaction mixture contained, in a final volume of 1 ml, 100 mM Tris (pH 8), 5 mM MgCl2, 100 \( \mu \)M 5,5'-dithio-bis-(2-nitrobenzoic acid), 33 \( \mu \)M acetyl-CoA, 10 mM sodium glyoxylate, and up to 100 \( \mu \)l of enzyme extract. The reaction was initiated by adding glyoxylate and was followed as an increase in A at 412 nm in a Gilford spectrophotometer. The A12 of the DTNB-coenzyme A complex formed was taken to be 13.6 cm2/\( \mu \)mol (26). One enzyme unit: \( \mu \)mol/min.

Choline phosphotransferase (EC 2.7.8.2), fumarase (EC 4.2.1.2), and isocitrate lyase (EC 4.1.3.1) were assayed as described previously (10, 20).

**Other Methods.** Protein was estimated by the method of Lowry (21) with BSA as standard. Sucrose concentrations were determined refractometrically. The radioactivity content of gradient fractions obtained during the purification of malate synthase from \[^{35}S\]methionine-labeled endosperm tissue was determined by counting samples (50 and 100 \( \mu \)l) of each fraction in 10 ml of Bray's (6) scintillation fluid. Vials were counted in a Packard Tri-Carb liquid scintillation counter at an efficiency of 60%.

**RESULTS**

**Separation of Cellular Organelles.** Sucrose density gradient centrifugation of homogenate prepared from endosperm tissue
Excised from 2-day-old seedlings effectively separated the major organelle fractions. The organelle marker enzymes cholinephosphotransferase (microsomes), fumarase (mitochondria), and isocitrate lyase (glyoxysomes) were recovered at mean buoyant densities of 1.12, 1.18, and 1.24 g/ml (Fig. 1a). The virtual absence of enzymic activity in the soluble phase at the top of the gradient (fractions 1–5) established that organelle breakage during homogenization and fractionation was minimal. When such gradients were assayed for a second glyoxysomal marker enzyme, malate synthase, a dual cellular location at this stage of tissue development was indicated, with a secondary but significant peak of activity being recovered at density 1.12 g/ml (Fig. 1b). This second malate synthase peak corresponded in its gradient distribution to the microsomal fraction, confirming the findings of Gonzalez and Beevers (14). Such gradients show that microsomal and glyoxysomal malate synthase activities are cleanly separated, and that activity is not associated with either the soluble or mitochondrial fractions.

**Purification of Malate Synthase from Isolated Organelles.** Gradient fractions containing peak activities of microsomal (fractions 8–12) or glyoxysomal marker enzymes (fractions 23–29) were pooled. The highly purified microsomal or glyoxysomal suspensions were used separately as starting material for the purification of malate synthase. The simple procedure employed took advantage of the earlier demonstration that whereas most glyoxysomal enzymes are readily solubilized when the organelles are deliberately disrupted by osmotic shock, malate synthase activity remains almost completely associated with the membranes (7). The organelle suspensions (in 0.1 M sucrose solution) were diluted with an equivalent volume of 50 mM Tricine buffer (pH 7.5) and the membranes subsequently recovered by centrifugation. Such washed membrane pellets contained 75 to 80% of the remaining malate synthase activity in the case of the microsomes and 85 to 90% in the case of glyoxysomes. Malate synthase was solubilized from these pellets by resuspending them in 0.2 M KCl (17); 90 to 95% of the membrane-associated activity was recovered in the supernatant after centrifugation. Attempts to fractionate malate synthase in such supernatants by ammonium sulfate treatment were unsuccessful, the enzyme apparently aggregated under such conditions. The final purification step employed sucrose density gradient centrifugation, a previous study having shown that the sedimentation rate of malate synthase in sucrose gradients is considerably greater than that of other glyoxysomal enzymes (7). Microsomal and glyoxysomal malate synthases sedimented at the same rate in sucrose gradients (Fig. 2a). Preliminary studies established that the inclusion of KCl in the sucrose solutions used to construct the purification gradients was essential to prevent aggregation of malate synthase. Typical enzyme purification data are shown in Table I.

The major limiting factor responsible for the low yield of malate synthase protein ultimately recovered was the difficulty in obtaining large amounts of isolated microsomes or glyoxysomes with the sucrose density gradient procedure used to fractionate cellular organelles from crude resuspended particulate fractions. In order to obtain malate synthase protein in sufficient quantity to permit antibody production in rabbits, purified glyoxysomes were isolated in much higher yields using a zonal rotor. In this way, and using the purification procedure outlined above, 1 to 2 mg of malate synthase could be obtained.

---

**Fig. 1.** Sucrose density gradient centrifugation of castor bean endosperm homogenates. An extract of 10 endospem halves from 2-day-old seedlings was applied to a sucrose gradient and centrifuged for 3 hr at 53,000g and C: a: Microsomes, mitochondria, and glyoxysomes were located on the gradient by the distribution of their respective marker enzymes: cholinephosphotransferase (●, nmol/hr [× 20]); fumarase (△, μmol/min [× 20]); isocitrate lyase (ollah, μmol/min [× 40]). b: Gradient distribution of malate synthase (○, nmol/min [× 5]) and sucrose concentration of sequential gradient fractions (---).
Purified malate synthase was incubated with SDS and subjected to electrophoresis in buffers containing SDS; a single protein band was visible on the stained gel. A typical densitometer trace of a gel containing glyoxysomal malate synthase is shown in Figure 6, a single band of identical mobility was also seen on gels containing the microsomal enzyme (data not shown). When such gels were calibrated with proteins of known monomer mol wt, the mol wt of the malate synthase monomer was estimated (five determinations) to be 64,000 ± 3,000. Accordingly, it appears that castor bean endosperm malate synthase is a multimeric enzyme containing several monomeric subunits.

Serological Comparison of Glyoxysomal and Microsomal Malate Synthases. Antiserum was raised in rabbits against purified glyoxysomal malate synthase. The whole antiserum thus obtained gave a single sharp precipitin band on Ouchterlony double diffu-
The resulting washed immunoprecipitate was subjected to SDS-polyacrylamide gel electrophoresis. Stained gels showed a single major protein band which migrated with an identical mobility as obtained with purified malate synthase (Fig. 8a). Gels were then sliced and the radioactivity present in each slice was determined. The bulk of the radioactive present on the gel occurred in a single radioactive peak with an identical mobility to that of the protein peak (Fig. 8b). The remainder of the gel contained only small amounts of radioactivity indicating that nonspecific precipitation or the adsorption of other protein species to the immunoprecipitate itself were of minor importance. Null serum precipitated negligible radioactivity.

**Kinetic and Inhibitor Studies.** All malate synthase assays were performed at pH 8 which was experimentally determined to be the pH optimum for the microsome and glyoxysome-derived enzymes, both of which showed an absolute requirement for Mg^{2+}. Purified microsomal and glyoxysomal malate synthases were identical in all kinetic parameters and inhibitor responses examined. The enzyme from either cellular location had an apparent $K_m$ for acetyl-CoA of 10 $\mu$M and for glyoxylate of 2 mM (Table II). In common with the enzyme from other sources (9, 11), malate synthase was inhibited by oxalate and glycolate (28). Lineweaver-Burk plots showed that the inhibition was competitive with respect to glyoxylate. Adenine nucleotides were also found to inhibit malate synthase activity, confirming the findings of Servettaz et al. (25) using the enzyme isolated from maize scutella (Fig. 9).

When added to the standard malate synthase assay mixture at final concentrations of 5 mM, AMP, ADP, and ATP resulted in 18, 46, and 55% inhibition, respectively. The inhibition caused by ATP was competitive with respect to acetyl-CoA (Fig. 10).

![Graph of mobility](image)

**Table II** Michaelis constants for glyoxysomal and microsomal malate synthase

<table>
<thead>
<tr>
<th>Substrate/substrate</th>
<th>Glyoxysomal malate synthase</th>
<th>Microsomal malate synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>10 $\mu$M</td>
<td>11 $\mu$M</td>
</tr>
<tr>
<td>Acetyl-CoA + ATP</td>
<td>15 $\mu$M</td>
<td>20 $\mu$M</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>2 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>Glyoxylate + ATP</td>
<td>25 $\mu$M</td>
<td>25 $\mu$M</td>
</tr>
<tr>
<td>Acetyl-CoA + Glyoxylate</td>
<td>40 $\mu$M</td>
<td>35 $\mu$M</td>
</tr>
</tbody>
</table>

Fig. 7. Inhibition of malate synthase activity by antiglyoxysomal malate synthase serum. Varying amounts of antiserum were added to 5 $\mu$g of purified glyoxysomal (O) or microsomal (O) malate synthase in final volumes of 0.2 ml. After 30 min at 25 C, aliquots of each mixture were removed and assayed for malate synthase activity. The control activities (no antiserum) were 8 to 9 nmol/min.

The enzymic activity of both glyoxysomal and microsomal malate synthase was inhibited by the antiserum to the glyoxysomal enzyme, the same inhibition-antibody titer relationship occurring (Fig. 7). Null serum had no effect on enzymic activity.

The antiserum was capable of specifically precipitating malate synthase from a mixture of proteins. This was tested by incubating the antiserum with a KCl extract prepared from whole glyoxysomes isolated from [$^{38}$S]methionine-labeled endosperm tissue.
Although it is clear that the criteria used here do not rigorously establish the homogeneity of our enzyme preparations, these preparations were regarded as being adequate for the most critical aspect of the current work. Such studies involved using the purified enzyme to raise antisera which itself was to be used in following the intracellular route of newly synthesized malate synthase as a model for the segregation of glyoxysomal enzymes (see accompanying paper). Since purified glyoxysomes and subsequently washed glyoxysomal membranes were taken as the source of malate synthase used to immunize rabbits, it seems reasonable to suggest that any contaminants in the purified enzyme, and hence in the antiserum eventually obtained, would be trace amounts of KCl-solubilized glyoxysomal matrix proteins. (The KCl concentration used is not effective in solubilizing integral membrane proteins [8]). Such contaminants would themselves be likely to follow the same intracellular route as malate synthase and their presence, if significant at all, should not complicate the over-all conclusions.

Regarding the significance of a microsomal as well as a glyoxysomal location of malate synthase in castor bean endosperm, particularly during the early stages of seedling development, we agree with the view of Gonzalez and Bevers (14) that this distribution may well offer a significant insight into the mechanism of glyoxysomal protein sequestration. Malate synthase present in the matrix of isolated glyoxysomes remains associated with the membrane vesicles if such organelles are deliberately broken (2, 17). It is tempting to suggest that microsomal malate synthase represents enzyme located in the ER cisternal space at the time of homogenization which likewise remains associated with the membrane vesicles derived from the ER during tissue homogenization. Other characteristic glyoxysomal enzymes, including those almost completely solubilized when the organelles are disrupted (for example, catalase), may also occur in the ER cisternae in vivo. Such conclusions are consistent with the accepted view that glyoxysomes arise from the ER by a process of vesiculation, their characteristic enzymes having been segregated initially by the ER membrane. The present demonstration that microsomal and glyoxysomal malate synthase are identical in all physical, chemical, and serological properties examined supports, but does not prove, this model. The complete absence of malate synthase from the soluble fraction after organelle separation makes it clear that more compelling evidence for this model could be provided if microsomal malate synthase could be shown to be ultimately recovered in the glyoxysomal fraction. Experiments which have confirmed this direction of cellular transport are described in the following paper.

Acknowledgment—We are grateful to L. K. Evans, Croda Premier Oils Ltd., Hull, U.K., for kindly providing the castor bean seeds.

LITERATURE CITED

4. HOCK B, H BEEVERS 1966 Development and decline of the glyoxylate cycle enzymes in watermelon seedlings (Citrus vulgaris Schrad.). Z Pflanzenphysiol 55: 405-414