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MALATE SYNTHASE FROM *GOSSYPIUM HIRSUTUM*

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Key Word Index—*Gossypium hirsutum*; Malvaceae; cotton; malate synthase; glyoxylate cycle; purification; kinetic analyses; reaction mechanism.

Abstract—Malate synthase was purified 2000-fold from cotyledons of dark-germinated cotton, *Gossypium hirsutum*. The purified enzyme had a pH optimum of 8.2, and an absolute requirement for a divalent cation. Only glyoxylate and acetyl-CoA served as condensation partners. Results obtained with functional-group directed inhibitors suggest the presence of lysine, tyrosine and histidine residues in the active site. Temperature optimum was 40°, and energy of activation was 3.3 kcal/mol. The MW of cotton malate synthase, determined by rate-zonal density gradient sedimentation, was 750 000. Initial-rate studies indicated Michaelis–Menten kinetics. Inhibition by substrate analogs, plus substrate-interaction kinetics gave results consistent with a sequential bireactant mechanism.

INTRODUCTION

Malate synthase (L-malate glyoxylate-lyase [CoA-acetylating], EC 4.1.2.3) catalyses the aldol condensation of acetyl-CoA and glyoxylate, in the presence of a divalent metal ion, yielding L-malate and CoA-SH. The reaction is irreversible under physiological conditions with a $\Delta G'_0$ of -12 kcal/mol [1]. Malate synthase is a component enzyme of the glyoxylate cycle, an anaplerotic pathway supplying biosynthetic intermediates in microorganisms [2], and is pivotal in gluconeogenesis from storage lipid during postgerminative growth of oilseeds [3]. Recently, it has been proposed that malate synthase may also play a role in organic acid metabolism in some bacteria [4] and in developing oilseeds [5].

Malate synthase has been purified from three different bacteria (*Escherichia coli* [6], *Bacillus stearothermophilus* [7] and *Pseudomonas ovalis* [1]) and the yeast enzyme has been purified and characterized extensively [8–14]. Purification from the alga *Euglena gracilis* has also been accomplished [15, 16]. Antibodies to the enzyme cross-reacted with malate synthase from acetate-grown *E. coli*, but not with enzyme from *Zea mays*, *Ricinus communis*, or acetate-grown *Chlorella fusca* [16].

Among higher plants, malate synthase is compartmented in specialized peroxisomes called glyoxysomes [3]. While glyoxysomes and the glyoxylate cycle have been studied extensively in germinated oil seeds, malate synthase has been purified and characterized only from *Ricinus communis* [17, 18], *Cucumis sativa* [19, 20] and *Zea mays* [21]. Maize malate synthase has a M_r of 480 000, while the enzymes from cucumber and castor bean are 540 000 and 575 000, respectively. In these higher plants, the enzyme apparently is an octamer of identical subunits. Recently, considerable evidence has accumulated suggesting that malate synthases from castor bean [22] and cucumber [20] are glycoproteins.

Although the stereochemistry of the malate synthase reaction has been examined thoroughly [10, 11], and much in the area of mechanistic organic chemistry has been accomplished with the yeast enzyme [12, 13], a detailed kinetic analysis has not been reported. In this paper we present data on the kinetics of malate synthase purified from cotton seeds. Such information on cottonseed malate synthase is important because it may have a different metabolic function in developing embryos than in germinated seeds [5, 23–25].

RESULTS

Malate synthase was purified *ca* 2000-fold to a sp. act. of 2.3 μkat/mg protein with 12% yield from cotyledons of cotton seeds germinated for 48 hr (Table 1). The purification scheme took advantage of enzyme subcellular localization, salt solubility, and size (MW).

The enzyme has a relatively sharp pH optimum at 8.2 in phosphate–citrate–borate buffer or in MOPS–KOH. In Tris–HCl, the pH optimum was 8. There was half-maximal activity at pH values of 6.8 and 9.7 in phosphate–citrate–borate buffer. Malate synthase activity was 2–3 times higher in MOPS or Tris than in phosphate–citrate–borate buffer.

The absolute requirement for divalent metal ions was best fulfilled with Mg^{2+}; *ca* 30% of this rate was observed with Ba^{2+}, Mn^{2+}, or Co^{2+}. An apparent K_m for Mg^{2+} of 0.59 mM was calculated from double-reciprocal plots of velocity vs Mg^{2+} concentration.

Malate synthase from cotton showed a high degree of substrate fidelity (Tables 2 and 3). Of numerous acids and aldehydes tested, only glyoxylate participated in the reaction (Table 2). Propionyl- and butyryl-CoA were slightly active as substrates (Table 3), but oxidized (acetoacetyl-CoA) or unsaturated (crotonyl-CoA) four

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Table 1. Purification scheme for malate synthase from cotyledons of 48-hr-old cotton seedlings

	Total units (nkat)	Total protein (mg)	Sp. act. (nkat/mg)	Yield (%)	Purification (fold)
S_1	1333	1176	1.1	100	1
P_2	885	290.0	3.1	66	3
S_3	830	29.3	28.3	62	26
DEAE supernatant	833	8.9	93.6	62	85
20–40% $(NH_4)_2SO_4$	418	2.2	190.0	32	173
5–20% sucrose gradient	163	0.07	2329	12	2117

Table 2. Glyoxylate analogs as substrates and inhibitors of *Gossypium hirsutum* malate synthase

	Substrate activity (%)	Inhibition (%)
Acetaldehyde	0	13
Glyoxylate	100	0
Glycolate	0	68
Oxalate	0	95
Oxamate	0	92
Pyruvate	0	11
Methylglyoxal	0	25

All compounds were tested as substrates at a final concentration of 1.5 mM, or as inhibitors at 5 mM in the presence of 1.5 mM glyoxylate. Inhibitors were preincubated with enzyme in assay medium for 1 min prior to substrate addition.

Table 3. Acetyl-CoA analogs as substrates and inhibitors of *Gossypium hirsutum* malate synthase

	Substrate activity (%)	Inhibition (%)
Acetyl-CoA	100	0
Propionyl-CoA	5	0
Butyryl-CoA	5	0
Chloro-acetyl-CoA	3	76
Dephospho-acetyl-CoA	14	38
Deamino-acetyl-CoA	0	88
Deuterated-acetyl-CoA	100	0
S-Acetonyl-CoA	0	100
S-Ethyl-CoA	0	100
ATP	—	53
ADP	—	24

All compounds were tested as substrates at a final concentration of 0.1 mM, or as inhibitors at 5 mM in the presence of 0.1 mM acetyl-CoA. Inhibitors were preincubated with enzyme in assay buffer for 1 min prior to substrate addition.

carbon acyl-CoAs were completely inactive. Chloro- and dephospho-acetyl-CoAs were slightly active as substrates, while deamino-acetyl-CoA was inactive. Activity with ^{2}H-acetyl-CoA (99 + atom % D) was equal to that with ^{1}H-acetyl-CoA.

Several glyoxylate analogs were inhibitors of malate synthase activity (Table 2). Oxalate and oxamate were highly inhibitory, as was glycolate. Acetaldehyde, pyruvate and methylglyoxal were slightly inhibitory. Formaldehyde, acetate, thioglycolate, propionate, bromopyruvate, benzaldehyde and phenylglyoxal were without effect on malate synthase activity. Among acetyl-CoA analogs, *S*-acetonyl-CoA, *S*-ethyl-CoA, chloroacetyl-CoA and deamino-acetyl-CoA were highly inhibitory.

Additionally, 5 mM ATP inhibited malate synthase more than 50%, while ADP was less inhibitory and AMP was without effect. GTP and ITP were less inhibitory than ATP. The analogs, 8-Br-ATP and 1,N^6-etheno-ATP were as inhibitory as ATP. Increasing Mg^{2+} concentration 10-fold did not ameliorate inhibition by nucleotides. Inhibition by ATP (or deamino-acetyl-CoA) was competitive with respect to acetyl-CoA (Fig. 2A) and noncompetitive with glyoxylate (Fig. 1A). Inhibition by glycolate (or oxalate) was competitive with respect to glyoxylate (Fig. 1B) and noncompetitive with respect to acetyl-CoA (Fig. 2B).

Measurement of reaction velocity as a function of substrate concentration resulted in rectangular hyperbolas for both glyoxylate (Fig. 3) and acetyl-CoA (Fig. 4). Double reciprocal plots were linear and Hill coefficients were 1 for both substrates. Apparent K_m values were 10 μM for acetyl-CoA and 52 μM for glyoxylate. V_{max}, calculated from replot of slopes vs reciprocal substrate concentrations (not shown) was 8.46 mkat/mg protein.

By holding glyoxylate concentrations constant and varying acetyl-CoA, a series of lines which intersect to the left of the *y*-axis of double reciprocal plots were obtained (Fig. 5A). Similar results were obtained when acetyl-CoA concentrations were held constant and glyoxylate varied (Fig. 5B).

A series of experiments were conducted using functional-group directed inhibitors (Table 4). *N*-Ethylmaleimide, *para*-chloromercuribenzoate and phenylmethylsulfonylfluoride were without effect on malate synthase activity. Butanedione was slightly inhibitory at high concentrations. Pyridoxal-5′-phosphate was very inhibitory, and this inhibition was

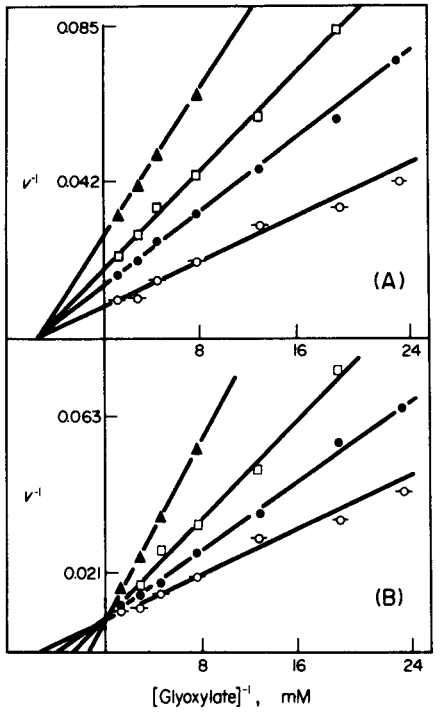

Fig. 1. Malate synthase activity as a function of glyoxylate concentration in the presence of: (A) 0 (-○-), 2.5 (●), 5.0 (□), or 12.5 (▲) mm ATP; or (B) 0 (-○-), 0.83 (●), 1.25 (□), or 2.5 (▲) mM glycolate.

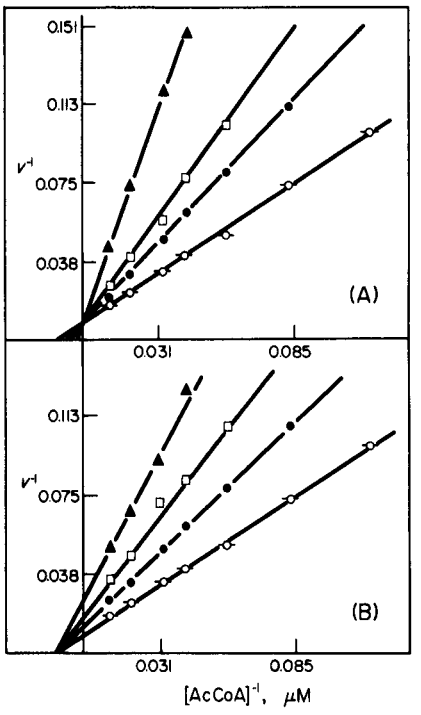

Fig. 2. Malate synthase activity as a function of acetyl-CoA concentration in the presence of: (A) 0 (-○-), 2.5 (●), 5.0 (□), or 12.5 (▲) mM ATP; or (B) 0 (-○-), 0.83 (●), 1.25 (□), or 2.5 (▲) mM glycolate.

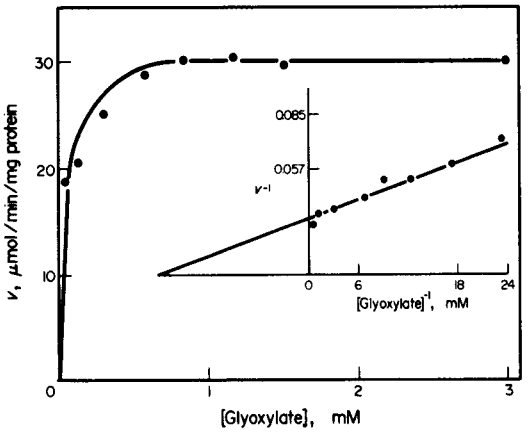

Fig. 3. Malate synthase specific activity as a function of glyoxylate concentration. Final acetyl-CoA concentration was 0.1 mM. Inset is a Lineweaver–Burk plot of the initial velocity data; K_m glyoxylate is 52 μM.

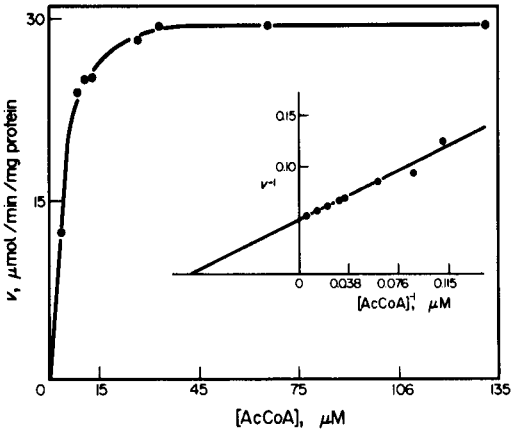

Fig. 4. Malate synthase specific activity as a function of acetyl-CoA concentration. Final glyoxylate concentration was 1.5 mM. Inset is a Lineweaver–Burk plot of the initial velocity data; K_m acetyl-CoA is 19 μM.

prevented by preincubation with glyoxylate, but not acetyl-CoA. Tetranitromethane also was inhibitory, and preincubation with either substrate protected against inactivation. Inhibition by diethylpyrocarbonate was prevented by preincubation with acetyl-CoA but not glyoxylate.

Purified cotton malate synthase was activated by Triton X-100 and was inactivated by deoxycholate. Tween 80, Lubrol W8, SDS, KCl and $NaClO_4$ were without effect.

Optimum temperature for the malate synthase reaction was 40°; 84 % of activity was lost after 2 min at 65°. A plot of log velocity vs temperature for values obtained between 25 and 40° resulted in a straight line, and using the slope of this line in the Arrhenius equation allowed calculation of an E_a of 3.3 kcal/mol.

M_r of malate synthase purified from cotton, determined by values from rate-zonal sucrose density-gradient centrifugation, was 750000 ± 8100, (n = 8) (Fig. 6). Gel filtration using a calibrated column of Bio-Gel A-15 yielded an M_r of 730000 (not shown).

DISCUSSION

Activity of cotton malate synthase was especially sensitive to pH and the type of buffer used in the

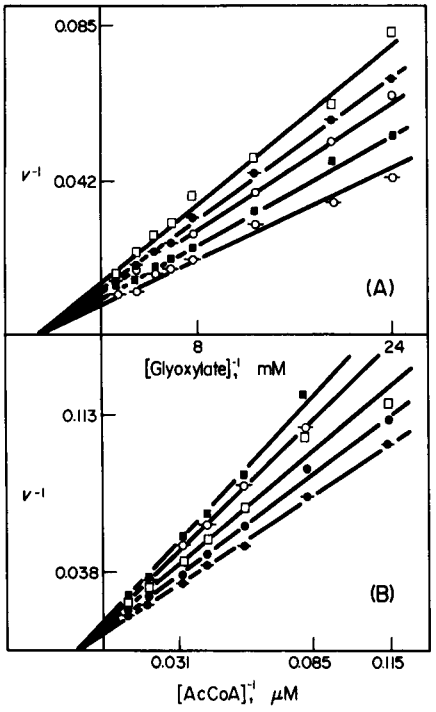

Fig. 5. Malate synthase activity with respect to: (A) glyoxylate in the presence of 0.131 (-○-), 0.066 (■), 0.049 (○), 0.033 (-●-) or 0.016 (□) mM acetyl-CoA; (B) acetyl-CoA in the presence of 1.5 (-●-), 1.0 (●), 0.5 (□), 0.25 (-○-), or 0.125 (■) mM glyoxylate.

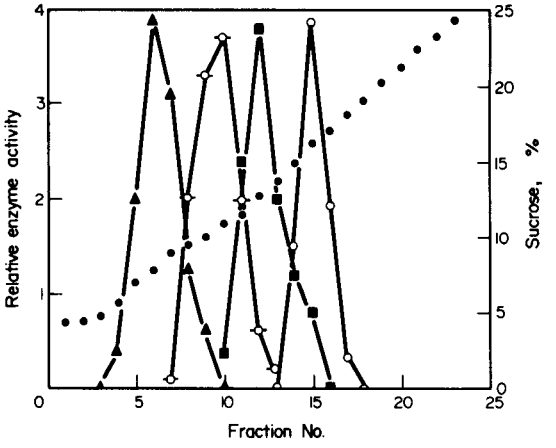

Fig. 6. Distribution of cotton malate synthase and marker enzymes on a sucrose-density gradient following centrifugation for 18 hr at 25 000 rpm in a Beckman SW27 rotor. Enzymes are: lactate dehydrogenase (▲), catalase (-○-), β-galactosidase (■), malate synthase (○).

homogenization medium. Highest activity and greatest stability were obtained with K_2HPO_4–KH_2PO_4, pH 6.9. Notably, lower activity was obtained with: glycylglycine, Bicine, Tris, Tricine, Hepes, Tes, imidazole, ACES, PIPES, ADA or MES (and at any other pH). Stability of malate synthase throughout purification was enhanced by Mg^{2+} and glyoxylate. Inclusion of glyoxylate was particularly important in the $(NH_4)_2SO_4$ step. Malate synthase recovered following rate-zonal sucrose density gradient centrifugation was stable for several months at 0°. A sp. act. of 2.3 μkat/mg protein is the highest recorded for malate synthase [1, 6, 16, 17, 19, 21].

Ethanol precipitation, column chromatography using Sephadex C-50 or agarose–hexane–CoA, and hydrophobic-interaction chromatography were ineffective in purifying cotton malate synthase. Ethanol (10%) inactivated malate synthase, and the enzyme bound irreversibly to C_6-agarose, suggesting a high degree of hydrophobicity.

No kinetic analysis of malate synthase has been reported. Values obtained from initial-rate studies of the cottonseed enzyme indicate Michaelis–Menten kinetics (Figs. 3 and 4). Mechanistic studies have been hindered by the apparent unidirectionality of the reaction [9]. Results using substrate analogs as inhibitors (Figs. 1 and 2), plus variable and fixed variable substrate studies (Fig. 5), are consistent with a sequential bi bi mechanism. Cotton malate synthase is not inhibited by L-malate (or the analogs malonate, maleate or itaconate) even at a concentration of 0.5 M, either in the presence or absence of CoA-SH. Additionally, we were unable to demonstrate transfer of tritium from malate to either acetyl-CoA or glyoxylate.

In common with most other sources [1, 12], malate synthase from cotton shows a high degree of substrate specificity (Tables 2 and 3). Virtually any change in glyoxylate (oxidation, reduction, elongation, substitution of sulfur or nitrogen for oxygen) renders it ineffective as a substrate. Slight substrate activity was found with acyl

Table 4. Effect of active site directed, functional-group inhibitors on activity of *Gossypium hirsutum* malate synthase

Inhibitor	Target	Inhibitor concn (% control) 1.0	0.5	0.1	0.01	Substrate protection Acetyl-CoA	Glyoxylate
N-Ethylmaleimide	Cysteine-SH	100	100	—	—	—	—
PCMB	Cysteine-SH	100	100	—	—	—	—
PMSF	Serine-OH	100	100	—	—	—	—
Butanedione	Arginine	82	91	100	100	no	no
Pyridoxal-5′-P	Lysine-ε-NH_3	0	0	19	51	no	yes
Tetranitromethane	Tyrosine	0	0	2	50	yes	yes
Diethylpyrocarbonate	Histidine	34	76	91	100	yes	no

PCMB — *p*-chloromercuribenzoate. PMSF — phenylmethylsulfonylfluoride.

Inhibitor was added to enzyme (0.17 nkat, 64 μg protein) in assay buffer; after 1 min, substrate was added and enzyme activity measured. In substrate protection studies, indicated substrate was added to cuvette 1 min prior to inhibitor addition. Malate synthase activity is given as per cent control.

groups larger than the acetate of acetyl-CoA. There are several reports of bacterial malate synthases which use acyl-CoAs other than acetyl-CoA, but reaction products may be unique to bacterial metabolism [26]. Both Dixon *et al.* [1], and Powell and Beevers [27] found that fluoroacetyl-CoA was slightly active as a substrate for malate synthase. Similarly, chloroacetyl-CoA showed activity with cotton malate synthase (Table 3). The bulk of halogenated derivatives may cause steric hinderance and interfere with substrate binding. Several structural aspects of acetyl-CoA binding can be deduced from analog studies (Table 3). Both the 3′-phosphate and N^6-amino group are necessary for efficient reaction. In contrast, dephosphoacetyl-CoA is neither a substrate nor an inhibitor of yeast malate synthase [28]. It seems likely that ATP inhibits malate synthase activity by competing for the adenine portion of the acetyl-CoA binding site. If this is true, then the 8-position of the purine ring is unimportant in CoA binding, since 8-Br ATP exhibited the same inhibition as ATP. Due to relatively high K_i values, it seems unlikely that inhibition by ATP or C_2 acids (Table 3) is significant in terms of physiological control of the enzyme.

Studies on yeast malate synthase [12] using ^{2}H-acetyl-CoA led to the conclusion that enolization, generation of a methylene group, and subsequent proton loss were rate limiting in the reaction. Results obtained with cotton malate synthase (Table 3 and unpublished) are in contrast to this proposal. ^{2}H-acetyl-CoA was as efficient a substrate as ^{1}H-acetyl-CoA, and apparent Michaelis constants and maximum velocities were identical. However, the requirement for substrate enolization was verified by the lack of activity with the nonenolizable analogs *S*-acetonyl-CoA or *S*-ethyl-CoA (Table 3). That these analogs were strong inhibitors suggests they were capable of binding to the substrate site. Hydrolysis of malyl-CoA by the yeast enzyme [1, 10] and by cotton malate synthase (not shown) was very slow, but it is not clear whether this was due to some limitation in catalysis, or merely slow binding of this proposed intermediate to the enzyme.

Results obtained with functional group inhibitors (Table 4), suggested that neither cysteine nor serine participated in substrate binding or catalysis. Inhibition by butanedione occurred only at high concentrations and substrate protection was not evident, implying that arginine was not directly involved. Inhibition by pyridoxalphosphate, tetranitromethane and diethylpyrocarbonate implied the existence of lysine, tyrosine, and histidine residues at the active site. As both substrates were protective against tetranitromethane, more than one tyrosine residue may be involved. Mid-points on the pH curve for cotton malate synthase closely correspond to p*K*s for the *R*-groups of histidine and tyrosine.

In studies concerned with glyoxysome characterization, malate synthase activity was stimulated by detergents or salts [3]. This activation may have been due to release of malate synthase from its association with the glyoxysome membrane, or change in conformation which led to increased activity. While most detergents and salts did not stimulate purified cotton malate synthase, addition of Triton X-100 caused a 16% activation.

With the exception of studies with thermophilic bacteria [7, 29], little has been reported on temperature optima for malate synthase. Cook [15] reported that partially purified enzyme from *Euglena* had a temperature optimum of 30° and an E_a of 12 kcal. A crude malate synthase preparation from castor bean endosperm glyoxysomes also had an E_a of 12 kcal/mol [30]. The temperature optimum for purified cotton malate synthase was 40° and the E_a was much lower at 3.3 kcal/mol. It is unclear whether these differences represent differences in degree of purification, or molecular dissimilarities between malate synthase from cotton and other sources.

Sundaram *et al.* [7] called attention to the differences in M_r values for malate synthase from various sources. The bacterial enzymes are relatively small and monomeric [1, 6, 7], while malate synthase from eucaryotic microorganisms are intermediate in size and either di- or tetrameric [14, 16]. Malate synthases from higher plants are comparatively large (MW > 500 000) and appear to be octamers of identical subunits [17, 19]. Malate synthase from cotton has the largest M_r yet reported. If it is an octamer, subunit MW would be near 100 000. Malate synthase from cucumber [31] forms a large aggregate with low sp. act. when exposed to solutions of low ionic strength. Since the ionic strength of the sucrose gradients used in this study was relatively high, it seems unlikely that the observed M_r was due to aggregation. Cotton malate synthase did aggregate at low ionic strength, but was irreversibly inactivated in contrast to results with the cucumber enzyme [31].

By several criteria, it has been shown that malate synthase from cucumber [20] and castor bean [22] are glycoproteins. Preliminary examination of cotton malate synthase has yielded equivocal results. Purified cotton malate synthase does not bind to immobilized Con A, nor does it react with lectins specific for fucose or galactose when tested by agar diffusion. Treatment of purified cotton malate synthase with β-glucosidase, neuraminidase, β-*N*-acetyl-glucosiminidase, α-galactosidase, α-mannosidase, β-xylosidase, α-glucosidase, α-L-glucosidase, α-L-fucosidase, or β-galactosidase, alone or in combination, was without effect on enzymatic activity. Furthermore, prior treatment with carbohydrases had no effect on the rate of trypsin inactivation of cotton malate synthase. However, treatment of cotton seeds with 0.2 mM bacitracin, an inhibitor of protein glycosylation [32], had no effect on germination, but almost completely inhibited development of malate synthase activity.

Previous reports from this laboratory have detailed development of malate synthase activity during cotton embryogenesis, and its persistence through seed desiccation [23, 24]. In contrast to its function in gluconeogenesis during postgerminative growth of oil seeds, Miernyk and Trelease [5] proposed that malate synthase in embryos plays a specific role in organic acid metabolism. Such distinct roles at different metabolic stages of plant development suggest that isoenzymes are involved. Micro-organisms grown on variable carbon sources have multiple forms of the enzyme [6, 33]. However, all chemical and enzymatic parameters examined for malate synthase from embryos and germinated seedlings were identical. Thus, it is unlikely that the distinct roles proposed in developing and germinated cotton seeds are attributable to multiple forms of the enzyme.

EXPERIMENTAL

Chemicals. Enzyme grade $(NH_4)_2SO_4$ and sucrose were from Schwartz-Mann. DEAE-Sephacel and Sephadex G-25 were from

Pharmacia. Acetyl-CoA and its analogs were prepared from CoA and various acid anhydrides as previously described [23]. *S*-Acetonyl-CoA was prepared according to ref. [34], and *S*-ethyl-CoA was prepared as described in ref. [35]. CoA and other acyl-CoAs were obtained from PL Biochemicals. Concns of acyl-CoA prepns were calculated from a mM extinction coefficient of 4.5 for the thioester bond at 232 nm [1]. All other chemicals were from Sigma.

Plants. G. hirsutum L. cv Deltapine 61, seeds were germinated and grown as previously described [24]. Seedlings grown for 48 hr were chosen as starting material because this stage had both highest total and sp. act. of malate synthase [24].

Malate synthase assay. Two different assays were employed. In most cases the previously described 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) assay was employed [23], but with substitution of MOPS–KOH buffer, pH 8.2, containing 0.05% (v/v) Triton X-100 as assay buffer. In cases where DTNB interfered, it was omitted from assays and decrease in A_{232} was recorded [1]. For all kinetic data, lines were generated by linear regression. Correlation coefficient of lines to the data points were 0.92 or better.

Purification of malate synthase. About 500 cotton cotyledon pairs in buffered osmoticum were chopped with razor blades attached to an electric knife as previously described [24, 25]. The homogenate was filtered through four layers of buffer-moistened Miracloth, then centrifuged at 522 *g* for 10 min in a Beckman JA-20 rotor to remove starch grains and cell debris. The supernatant (S_1) was further centrifuged at 19 300 *g* for 30 min to obtain a glyoxysome-enriched pellet (P_2). This pellet was resuspended in 5 ml of 200 mM K–Pi, pH 6.9, containing 0.6 M $MgCl_2$ and 2 mM Na glyoxylate, and passed twice through a French pressure cell (American Instrument Co.) at 984 kg/cm^2. The pressate was then centrifuged at 100 000 *g* for 90 min in a Beckman Ti-50 rotor in a Beckman L5-65 ultracentrifuge, and the supernatant (S_3) taken for further purification. S_3 was dialysed overnight against 50 mM K–Pi, pH 6.9, containing 12 mM $MgCl_2$ and 2 mM sodium glyoxylate. After clarification by centrifugation (19 300 *g*, 20 min), dialysed S_3 was mixed with an equal vol. of DEAE-Sephacel and held at 4° for 60 min with intermittent mixing. Sephacel beads were removed by centrifugation and the supernatant fractionated with solid $(NH_4)_2SO_4$. The 20–40% (w/v) ppt. was collected by centrifugation, dissolved in a small vol. of dialysis buffer, and passed through a Sephadex G-25 column (2 × 14 cm) equilibrated with the same buffer. Malate synthase in the void vol. was subjected to rate-zonal sucrose density gradient centrifugation as described in ref. [36]. All sucrose solns were made up in 100 mM K–Pi, pH 6.9, containing 12 mM $MgCl_2$, 50 mM KCl, and 2 mM Na glyoxylate. Malate synthase soln (1 ml) (0.3 mg protein) was applied to each 14.5 ml, 5–25% (w/w) linear sucrose gradient, and centrifuged at 25 000 rpm for 18 hr using 16 × 102 mm tubes in a Beckman SW-27 rotor. Fractions (0.6 ml) were collected from the top by pumping 45% (w/w) sucrose into the bottom of the tube.

Protein was determined by the method of ref. [37].

Divalent metal requirement. Malate synthase purified through $(NH_4)_2SO_4$ fractionation was dialysed against 100 mM K–Pi, pH 6.9, containing 5 mM Na_2EDTA, then assayed with various metal ions at final concns of 5 mM. The final EDTA concn in these assays was 25 μM; all calculations were corrected for this concn.

Substrate specificity and inhibitors. All acid substrates were tested at a final concn of 1.5 mM, and all acyl-CoAs at 0.1 mM. Substrates were incubated with enzyme in reaction buffer for 1 min before addition of the second substrate. Similarly, substrate analogs used as inhibitors were incubated with enzyme in reaction buffer for 1 min before addition of glyoxylate or acetyl-CoA. Active-site directed inhibitors were tested over a range of 0.1–1 mM, with a 1-min preincubation.

Effects of detergents and salts on malate synthase activity. Enzyme purified through $(NH_4)_2SO_4$ fractionation was preincubated (20 min) and assayed with detergent at 1% (v/v), or salts at 0.2 M.

Temperature optimum. Assays were conducted using a thermostatted cuvette holder. Malate synthase activity as a function of temp. was measured at 5° increments from 20 to 50° following a 5-min equilibration at a given temp.

MW Determination. The rate-zonal centrifugation method of ref. [36] as described for purification of malate synthase was used. Markers of known M_r, e.g. beef heart lactate dehydrogenase (136 700), beef liver catalase (250 000), and *E. coli* β-galactosidase (540 000) were used to calculate M_r for cotton malate synthase.

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