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Relationship between Cottonseed Malate Synthase Aggregation Behavior and Suborganellar Location in Glyoxysomes and Endoplasmic Reticulum[1]

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ABSTRACT

Malate synthase (EC 4.1.3.2) (MS), an enzyme unique to the glyoxylate cycle, was studied in cotyledons of dark-grown cotton (*Gossypium hirsutum*, L.) seedlings. MS has generally been regarded as a peripheral membrane protein in glyoxysomes and believed by some to be synthesized on rough ER. Immunocytochemical localization of MS in both *in situ* and isolated cottonseed glyoxysomes, however, showed that MS was located throughout the matrix of glyoxysomes, not specifically associated with their membranes. Biochemical data also supported matrix localization. Isolated glyoxysomes were diluted in variously-buffered salt solutions (200 millimolar KCl or 100 millimolar K-phosphate) or detergents (0.1% Triton X-100, 10 millimolar deoxycholate, or 1.0% Triton X-114) and centrifuged to pellet membranes. Greater than 70% of the MS was recovered in supernatants after treatment with salt solutions, whereas generally less than 30% was released following detergent treatments. MS in pellets derived from glyoxysomes burst in low ionic strength buffer solutions was aggregated (observed on rate-zonal gradients). MS released following salt treatments was the 20S nonaggregated form indicating that salt solutions either disaggregated (or prevented aggregation of) glyoxysomal MS rather than releasing it from membranes. We confirmed reports by others that MS comigrated with ER (NADH: cytochrome *c* reductase) in sucrose (20–40% w/w) gradients buffered with 100 millimolar Tricine (pH 7.5) after 3 hours centrifugation. However, cottonseed MS did not comigrate with ER in gradients buffered with 10 millimolar Hepes (pH 7.0) or 20 millimolar K-phosphate (pH 7.2) after 3 hours centrifugation, or after 22 hours centrifugation in Tricine or Hepes. Collectively, our data with cotton seeds indicate that MS is not a peripheral membrane protein, and that the aggregation behavior of MS (in various buffers) very likely has led to misinterpretations of its putative associations with ER and glyoxysomal membranes.

Malate synthase is housed in specialized organelles called glyoxysomes and functions in the glyoxylate cycle during heterotrophic growth of oilseeds. MS[2] has generally been considered to be a peripheral membrane protein in glyoxysomes (1, 4, 15, 16, 23, 27) with few exceptions (22, 26), and consequently has gained acceptance as a marker enzyme used to help define the suborganellar localization of other glyoxysomal enzymes (3, 14, 15, 17, 27). This generalization is based largely on studies where MS was purified from glyoxysomal membrane fractions (*e.g.* 1, 4, 17, 25), and on interpretations of data showing MS released from isolated glyoxysomes diluted in buffered salts (150 mM KCl, 100 mM Na_2CO_3, 200 mM KCl, 100 mM $MgCl_2$), but not in buffer solutions alone (5, 8, 13, 17, respectively). In addition, the 5S cytosolic monomer, and to a lesser extent the 19S glyoxysomal octamer of MS in cucumber cotyledons were shown to have binding affinity for phospholipids (20), and MS was shown to comigrate with membrane 'ghosts' from lysed castor bean (13) and peanut (14) glyoxysomes.

Alternative interpretations of MS localization, however, have been made from enzyme cytochemical (30) and immunocytochemical (25, 29) studies which showed enzyme reaction product and MS protein, respectively, distributed throughout the glyoxysomal matrix, not specifically associated with membranes. Fang *et al.* (8), postulated a matrix location for MS in castor bean endosperm, and Mori *et al.* (25) suggested that pumpkin seed MS was not associated with the glyoxysomal membranes *in vivo*. In none of the cases above, however, did the researchers reconcile the apparent discrepancies.

MS has been identified by at least two groups (10, 20) as an aggregate in ER fractions collected from sucrose gradients. However, contrasting interpretations were made as to the location of the MS in these fractions, *i.e.* either inside (10, 23) or outside (16, 19) of the ER vesicles. If MS were inside the vesicles, then the interpretations were that MS was sequestered within the ER lumen via cotranslational insertion mechanisms prior to budding and forming nascent glyoxysomes. Alternatively, MS sedimented in the gradients as an aggregate independent of the vesicles, unrelated to MS biosynthesis or glyoxysome biogenesis.

The purpose of this study was to reconcile the apparent conflicting results and/or interpretations of the localization of MS in glyoxysomes and ER of germinated oilseeds. Clarification of suborganellar and subcellular location of MS is important to ultimately understanding the organization of catalytic reactions within glyoxysomes and for deciphering the mechanisms of intracellular targeting and import of MS into glyoxysomes. This work has been presented as a preliminary report with other authors (6).

MATERIALS AND METHODS

Chemicals

Potassium phosphate, potassium ferricyanide, potassium chloride, magnesium chloride, EDTA, sucrose, lead nitrate,

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[2] Abbreviations: MS, malate synthase; ICL, isocitrate lyase; CCR, NADH:Cyt *c* reductase; FCR, NADH:ferricyanide reductase; PBS, 10 mM Na phosphate, 0.9% w/v NaCl, pH 7.2; DOC, deoxycholate.

sodium citrate, and uranyl acetate were purchased from J. T. Baker Chemical Co. Proteinase K, isocitrate, NADH, Cyt *c*, Tricine, Hepes, trypsin, DOC, Triton X-100, were purchased from Sigma Chemical Co. Papain was from Calbiochem. LR White (hard grade) was from Ernest Fullam, Inc. Protein A-gold (colloidal 15 nm) was from E Y Labs, Inc. Triton X-114 was from Serva Fine Biochemicals, Inc. Glutaraldehyde (25%) was from Electron Microscopy Sciences, Inc., and paraformaldehyde was from Allied Chemical Co.

Plant Material

Cotton seeds, *Gossypium hirsutum*, L. cv Coker 100 (glandless) were soaked in distilled water with aeration 4 h (30°C), then scrolled in moistened filter paper for germination and growth in the dark at 30°C (21).

Isolation of Glyoxysomes and ER

Glyoxysomes were isolated from cotyledons of 2-d dark-grown cotton seedlings. Cotyledons (120, approximately 9 g fresh weight) were chopped into approximately 1 to 2 mm segments using a modified electric knife (21) in 20 mL medium containing 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 400 mM sucrose, and one of the following buffers: 50 mM K-phosphate (pH 7.2), 50 mM Hepes (pH 7.0), or 100 mM Tricine (pH 7.5). The homogenate was filtered through one layer of Miracloth (presoaked with homogenizing medium) and centrifuged 10 min at 480*g* (4°C) in a Beckman JS-13 rotor. The supernatant was then centrifuged in the same rotor for 20 min at 19,000*g* (4°C) to obtain a glyoxysome-enriched pellet (3). The pellet was resuspended in 3 mL homogenizing medium, layered onto a 20 to 56% w/w linear sucrose gradient (32 mL on a 5 mL 60% w/w cushion) buffered with either 20 mM K-phosphate (pH 7.2), 10 mM Hepes (pH 7.0), or 100 mM Tricine (pH 7.5) (in accordance with homogenizing buffer), and centrifuged 45 min (4°C) at 24,500 rpm (50,000*g*) in a Beckman VTi-50 rotor.

Endoplasmic reticulum was isolated from cotyledons of the same age. The same variations in homogenizing media were used as above except $MgCl_2$ was deleted in all cases. Both 10 mM KCl and 1 mM EDTA were included in buffers used in the gradients. Cotyledons (90, approximately 6 g fresh weight) were homogenized in 5 mL medium and a 480*g* supernatant was prepared as described above. The supernatant (about 5 mL) was layered directly onto a sucrose gradient (20–40% w/w, 24 mL on a 3 mL 45% w/w cushion) and centrifuged in a Beckman SW 25.1 rotor at 20,000 rpm (45,000*g*) for 3 h or 22 h. All procedures were performed at 4°C.

Organelles were collected in 1.2 mL fractions using an ISCO model 640 Density Gradient Fractionator. Malate synthase (31), catalase (21), and ICL (3), CCR (12), and FCR (12) were assayed as previously described. Antimycin A (0.002 mM) and KCN (0.2 mM) were included in CCR assays to ensure ER specificity. Sucrose concentration was determined using a Bausch and Lomb Abbe 2C refractometer.

Disruption of Glyoxysomes

Glyoxysomes (2–3 fractions with coincident peak MS, ICL, and catalase activities in about 53% w/w sucrose) were pooled and diluted with two volumes of treatment buffer (Table I). In each case the buffer was the same as used in sucrose gradients. The samples were vortexed and allowed to stand 30 min (22°C), then centrifuged at 100,000*g* for 30 min (4°C) in a Beckman Ti 70.1 rotor. Pellets, resuspended in 1 mL of respective treatment buffer, and supernatants were assayed for enzyme activities and protein content (Coomassie blue dye-binding method using bovine plasma γ-globulin as a standard). Percent solubilization of protein and enzymes were expressed as the amount remaining in the supernatant compared to the total in the supernatant and pellet multiplied by 100 as done by others (3, 13, 22).

Phase Separation with Triton X-114

Glyoxysomes were isolated as above and fraction(s) (one or two) exhibiting peak MS activity were diluted with six volumes of 1% (v/v) Triton X-114 in the same buffer as used for the gradient. Samples were incubated 20 min on ice, then warmed at 30°C for 3 min in a dry heat block according to Bordier (2). The large micelles that formed were sedimented in a 15-mL conical centrifuge tube at 1,890*g* (setting 7) for 6 min in a MSE GT-2 tabletop centrifuge (22°C). Enzyme activities were determined in the micelle phase (pellet) and the aqueous phase (supernatant). The supernatant was centrifuged using the same conditions as for the disruption experiments described above (100,000*g*, 60 min).

Rate-Zonal Centrifugation of Released Glyoxysomal MS

Rate-zonal centrifugation and calculations of S values were done essentially as in Trelease *et al.* (31), except linear gradients were 17 to 45% w/w sucrose in the various buffers with or without KCl.

Electrophoretic (Western) Blotting

Blotting from SDS-PAGE gels and immunodetection was accomplished as described in detail by Kunce and Trelease (21), except with anti-MS serum as done by Turley and Trelease (32).

Electron Microscopy

Cotyledons from 2-d-old dark-grown seedlings were sliced into 2 mm square segments in 2.0% (v/v) formaldehyde (prepared fresh from paraformaldehyde; see 30), 2.5% (v/v) glutaraldehyde, 50 mM K-phosphate (pH 6.9), and fixed in the same solution for an additional 1.5 h at 4°C. Segments were dehydrated in a graded ethanol series through 95% ethanol followed by three 15 min changes in 100% ethanol. The samples were then infiltrated with three changes of fresh LR White resin (1 h at 22°C, overnight at 4°C, then 1 h 22°C), placed in gelatin capsules containing fresh resin and polymerized 24 to 48 h at 57°C. Isolated glyoxysomes were fixed near isoosmotically in 53% w/w sucrose, 0.8% (v/v) formaldehyde, 1.0% (v/v) glutaraldehyde, 25 mM K-phosphate (pH 7.2) for 30 min (4°C). The glyoxysomes were then diluted in the same fixative solution without sucrose by adding ten volumes, 1 mL at a time (with slow inversions) over a 30 min period at 22°C. The fixed material was collected by centrifu-

Table I. *Percent Solubilization of MS, ICL, and Protein from Isolated Cottonseed Glyoxysomes following Osmotic Breakage in Three Buffers Containing Various Salts and Detergents*

Treatment	K-Phosphate (20 mM)			Hepes (10 mM)			Tricine (100 mM)		
	MS	ICL	Protein	MS	ICL	Protein	MS	ICL	Protein
					%[a]				
Buffer alone	17	97	59	23	96	42	19	97	27
0.2 M KCl	97	92	78	85	92	68	92	100	69
0.1 M K-Phosphate	91	96	73	73	89	72	ND[b]	ND	ND
0.1% Triton X-100	13	99	54	3	100	31	17	98	47
10 mM DOC	8	100	ND	30	97	ND	59	99	ND

[a] Supernatant values divided by supernatant + pellet values × 100. [b] Not determined.

gation (19,000*g* for 20 min, 4°C) onto a Gelman GA-6 filter (13 mm, 0.2 μm pore size) placed at the bottom of a 15 mL corex tube. The filter (layered with glyoxysomes) was covered with a drop of 5% (w/v) agar, then dehydrated in ethanol and infiltrated with LR White resin as above. After infiltration was complete, the agar was carefully separated from the filter (the glyoxysome layer was retained in the agar), then embedded and polymerized in LR White resin as above. Silver-gold sections were cut with a glass knife on a Sorvall MT-2B ultramicrotome and transferred to naked 300 mesh nickel grids. Immunolocalization of MS in sections was done as previously described (7) except 0.5% Carnation nonfat dry milk (instead of BSA) was used as the blocking agent and to dilute the protein A-gold and IgG solutions. This modification was optimized by Dr. Francisco Carrapico using sections of *in situ* glyoxysomes (6). Affinity-purified IgGs were prepared as described in Trelease *et al.* (31).

Briefly, the grids were floated on a 15 μL drop of an antibody dilution for 3 h (1:30 in PBS/milk), washed, then floated on a 15 μL drop of a Protein A-gold dilution (15 nm, 1:30 in PBS/milk) for 1 h. Sections were poststained in 2% aqueous uranyl acetate (3 min), followed by Reynold's lead citrate (1 min) and examined at 60 or 80 kV in a Philips EM300 transmission electron microscope.

Proteolysis of ER fractions

The peak CCR/MS fraction (1.2 mL, 27% w/w sucrose) from gradients buffered with Tricine (3 h centrifugation) was incubated with one volume of a mixture of Proteinase K, trypsin, and papain (0.6 mg/mL each) in 100 mM Tricine, 10 mM KCl, 1 mM EDTA (pH 7.5) and having the following variations: (a) 27% (w/w) sucrose to maintain vesicles, (b) 27% sucrose and 0.5% (v/v) Triton X-100 to solubilize vesicles, or (c) no sucrose or Triton X-100 to burst vesicles. FCR, CCR, and MS activities were assayed before and after 1 h incubation at 37°C.

RESULTS

Electron Microscopic Observations

Immunocytochemical localization of MS in thin sections of *in situ* and isolated glyoxysomes from cotyledons of cotton seedlings showed MS distributed specifically throughout the matrix of the organelles rather than preferentially in or nearby their membranes (Fig. 1). The similarity in the immunocytochemical labeling pattern of glyoxysomes fixed within cells or isolated in 53% (w/w) sucrose, indicated that the isolation procedure did not alter the suborganellar location or distribution of MS. Glyoxysomes isolated in Hepes or Tricine buffered gradients (not shown) exhibited the same immunocytochemical labeling pattern as those shown in Figure 1B. There was no apparent labeling of profiles or surface views of rough or smooth ER in the cotyledon cytoplasm. This cannot be construed as evidence against ER localization because only a minute amount would be present at any one time. Application of preimmune serum or anti-MS antibodies preadsorbed with purified MS protein validated the staining shown in the glyoxysomes in Figure 1, A and B.

Characteristics of Glyoxysomal MS

Only a small proportion of MS (not greater than 25%) was solubilized from glyoxysomes by any of the buffer treatments alone, whereas essentially all of the ICL activity was released (Table I). MS was almost completely solubilized by high salt treatments in all three buffers tested. Detergents, used in concentrations well above their critical micelle concentrations, were generally ineffective in releasing MS (Table I). ICL was completely solubilized by dilution in salt or detergent treatments in all buffers tested (Table I). Recoveries of MS and ICL activity after centrifugation were greater than 85% except for the Hepes-buffered DOC treatment (64% recovery).

Phase separation of material in glyoxysome fractions using the detergent Triton X-114 resulted in varying amounts of MS activity being partitioned into the aqueous phase depending on the buffer used (Table II). In contrast, ICL activity was recovered entirely in the aqueous phase. This aqueous (nonmicellar) phase was centrifuged at 100,000*g* (as were the samples shown in Table I). In Hepes and Tricine, most of the MS activity (87 and 88%, respectively) was recovered in the 100,000*g* pellets (Table II), indicating that MS was aggregated and sedimented under these conditions, independent of the presence of membranes. In K-phosphate, however, the majority of the MS activity was recovered in the 100,000*g* supernatant. ICL activity was recovered entirely in the 100,000*g* supernatant in all buffers tested.

Figure 2 shows results of rate-zonal centrifugations of various fractions in Tricine buffer. Results were essentially identical with K-phosphate and Hepes and therefore are not shown. MS in the membrane pellets (after bursting glyoxysomes in buffer alone) was aggregated (Fig. 2B). The MS solubilized in 200 mM KCl (Fig. 2A) consisted only of the

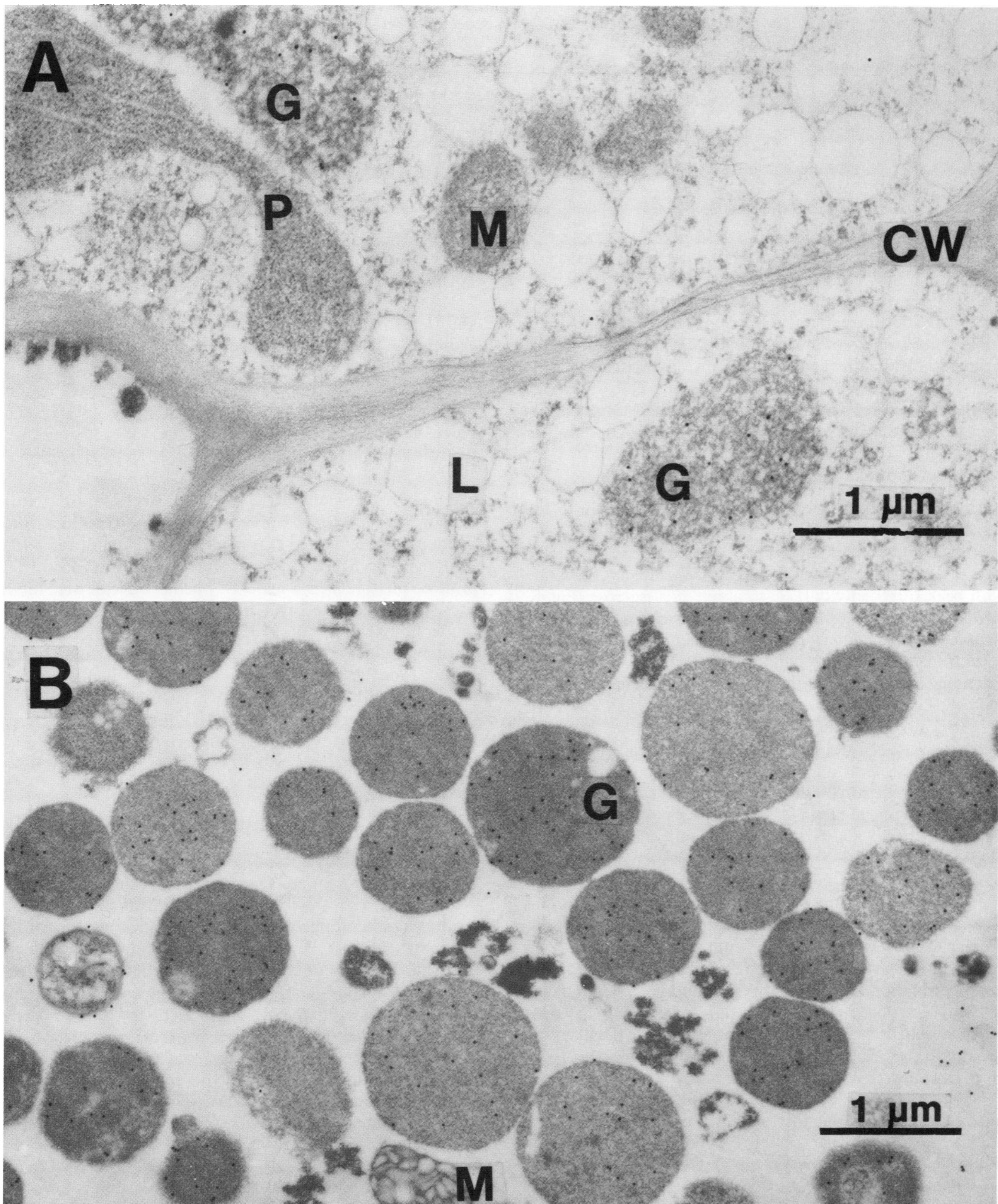

Figure 1. Electron micrographs of (A) a section through a cotton cotyledon cell from 2-d-old dark-grown seedlings and (B) a representative section of glyoxysomes isolated in a K-phosphate buffered sucrose gradient. Sections of both samples were immunocytochemically stained for MS protein using an indirect protein A-gold method. Colloidal gold particles are distributed over the matrix of the glyoxysomes (G) in both cases, but not over cell wall (CW), plastids (P), mitochondria (M), or lipid bodies (L).

Table II. *Percent Solubilization of MS and ICL from Isolated Cottonseed Glyoxysomes following Phase Separation in Triton X-114*

Fraction	K-Phosphate (20 mM)		Hepes (10 mM)		Tricine (100 mM)	
	MS	ICL	MS	ICL	MS	ICL
			%[a]			
Aqueous phase 1,890*g* spin	65	100	95	95	43	98
Supernatant 100,000*g* spin	63	100	13	100	12	96

[a] Supernatant values divided by supernatant + pellet values × 100.

20S, native dodecameric (31, 32), nonaggregated form. Rate-zonal centrifugation of the lipid-free 100,000*g* pellet from the Triton X-114 treatments revealed only aggregated MS (Fig. 2C). However, when this same lipid-free pellet was resuspended in buffered KCl (200 mM), all of the MS was disaggregated into the 20S form (Fig. 2D).

Relationship of MS and ER in Sucrose Gradients

The centrifugation behavior of MS varied relative to the equilibration density of ER (CCR) in sucrose gradients, depending on the buffer used (Figs. 3 and 4). Only in Tricine after 3 h centrifugation did a peak of MS activity comigrate with peak CCR activity (ER) to about 27% sucrose (Fig. 3, top panel). A peak of MS activity also was observed in the soluble-protein region (top) of this gradient. In Hepes after 3 h centrifugation, a broadband of MS activity was observed extending from the top of the gradient to a peak at about 23% sucrose (Fig. 3, middle panel). A single, broad CCR peak appeared at 27% sucrose. In 20 mM K-phosphate, after 3 h centrifugation, MS did not migrate into the gradient (Fig. 3, bottom panel). Two major peaks of CCR activity were observed under these conditions, one at the sample/gradient interface and one at about 27% sucrose, the same position as ER in the Tricine and Hepes buffered gradients.

After 22 h centrifugation in a Tricine-buffered gradient, a peak of MS activity did not comigrate with CCR as it did after 3 h (compare top panels Figs. 3 and 4). Instead, one peak appeared in the 34% sucrose region and another at about 23% sucrose, without any significant MS at the top of the gradient as observed after 3 h centrifugation. Essentially the same distribution of MS activity was found in Hepes-buffered gradient centrifuged for 22 h (Fig. 4, bottom panel). Samples in K-phosphate were not centrifuged for 22 h because MS was not in the ER region after 3 h (Fig. 3, bottom panel). In both Tricine and Hepes, peak CCR activity remained in the 27% region of the gradients (Fig. 4). Thus, it appears that ER was at equilibrium in approximately 27% sucrose, while MS migrated in a rate-zonal fashion depending upon the aggregation state of MS in the various buffers.

To determine whether inactive MS (possibly due to isolation conditions) was located in the ER region, gradient fractions were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with anti-MS serum (Fig. 5). The intensity of staining for MS protein corresponded well to profiles of MS activity in the sucrose gradients. Heavy staining occurred in the 27% sucrose region of the Tricine and Hepes blots when samples were taken after 3 h centrifugation (Fig. 5, A and C), whereas MS protein was essentially undetectable in this region after 22 h centrifugation (Fig. 5, B and D). MS was detected in the 23 and 34% sucrose regions of the gradients, corresponding to activity peaks shown in Figure 4. These results strongly indicated MS, active or inactive, did not cosediment with ER after 22 h centrifugation.

In an attempt to assess whether the MS present in the ER region in Tricine-buffered sucrose gradients (after 3 h centrifugation) was located inside or outside the ER vesicles, peak fractions were subjected to proteolytic treatments (Table III). Essentially no MS activity remained in the presence of the protease mixture after 1 h (37°C) whether vesicles were intact (in sucrose), disrupted by Triton X-100, or osmotically burst (buffer only), indicating that MS was not protected from proteolytic attack by a boundary membrane. Assays were included for ER electron transport enzymes (membrane proteins, FCR, and CCR). FCR activity was unchanged in sucrose and in the presence of Triton X-100, but only 52% activity remained after treatment with a hyperosmotic protease mixture (buffer only). Only 15% CCR activity remained in treatments with or without Triton X-100 (in sucrose).

DISCUSSION

The suborganellar localization of MS in oilseed glyoxysomes has been examined microscopically using both enzyme cytochemistry (30) and immunocytochemistry (25, 29). These results were interpreted to indicate a matrix localization of the enzyme *in vivo*. Biochemical data obtained with isolated organelles (see later discussion), however, indicated a 'peripheral membrane' association for MS in glyoxysomes. In discussions of these conflicting interpretations (25, 30), it was suggested that isolation of the organelles, or buffers used, could somehow cause an artificial association of MS with the boundary membrane. We isolated cotton cotyledon glyoxysomes in sucrose gradients made with three of the most common buffers used to isolate glyoxysomes (Table I). The immunogold localizations clearly showed that MS was distributed in the matrix of isolated organelles prepared in all three buffer systems as it was in *in situ* glyoxysomes. A distinct localization of membrane protein(s) can be observed in immunocytochemically prepared sections (9); this was not apparent in our micrographs. While the possibility exists that membrane bound MS was not recognized by the antibodies on sections, cytochemical localization via enzyme reaction (30) did not reveal a membrane localization.

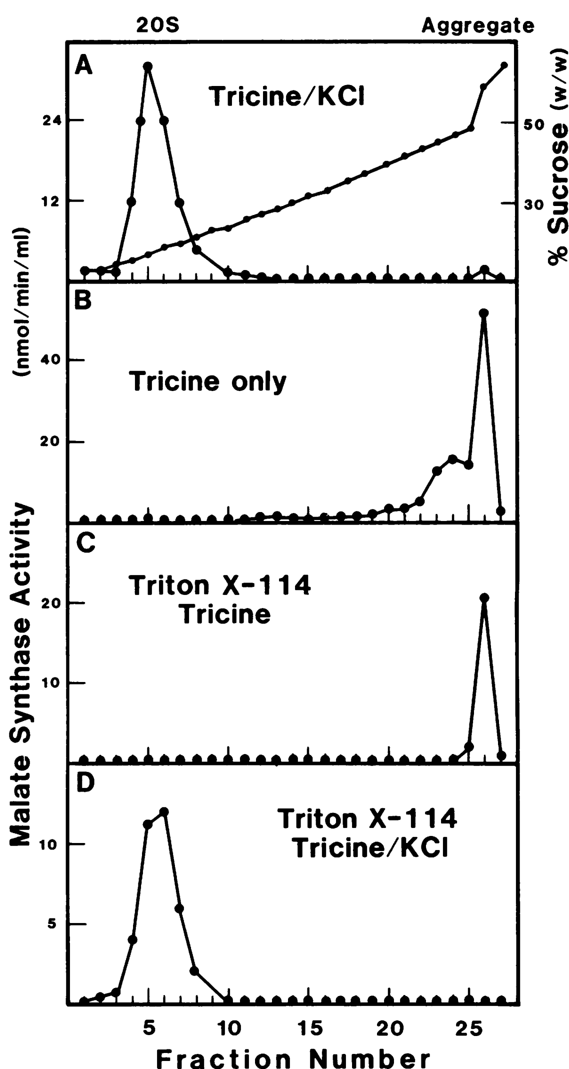

Figure 2. Profiles of MS activity following rate-zonal centrifugation of supernatants and resuspended pellets from hyperosmotic treatments of glyoxysomes isolated in 100 mM Tricine-buffered (pH 7.5) sucrose gradients. A, The sample was from the 100,000*g* supernatant of glyoxysomes diluted in 100 mM Tricine, 200 mM KCl (pH 7.5) (100% recovery of MS in the gradient); B, the sample was the 100,000*g* pellet of glyoxysomes isolated in 100 mM Tricine (pH 7.5) (90% recovery). In C and D, the samples were from the Triton X-114 phase separation experiments. The 100,000*g* pellet from the aqueous phase was resuspended in (C) 100 mM Tricine (pH 7.5), (61% recovery), or (D) 100 mM Tricine, 200 mM KCl (pH 7.5) (62% recovery). Samples were layered onto 17 to 45% w/w sucrose gradients with or without KCl.

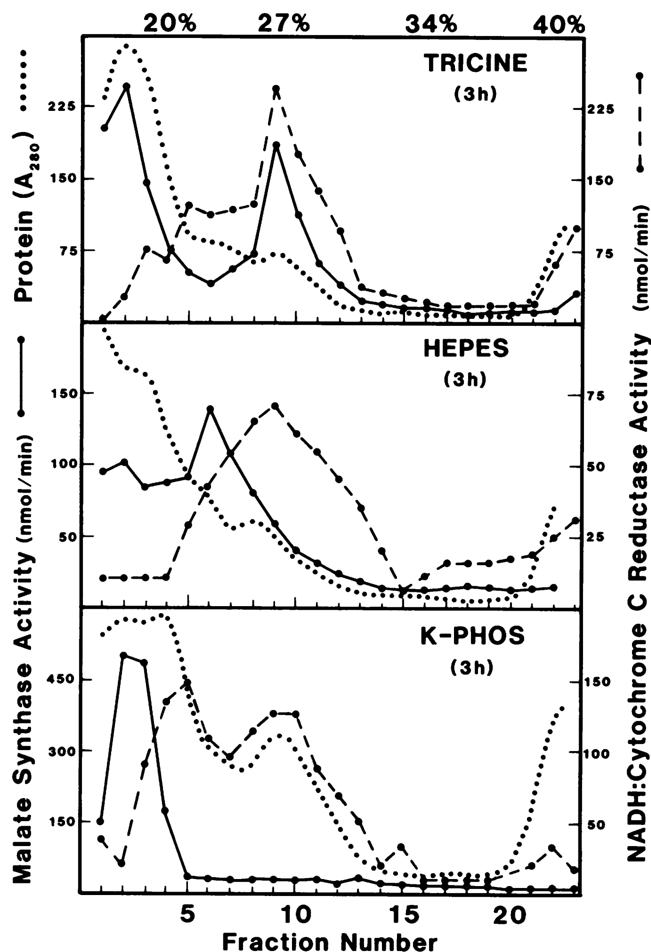

Figure 3. Centrifugation behavior of malate synthase (MS), NADH:Cyt *c* reductase (CCR), and protein in linear sucrose gradients (percent w/w given at top), in 100 mM Tricine (pH 7.5) (top panel), 10 mM Hepes (pH 7.0) (middle panel), and 20 mM K-phosphate (pH 7.2) (bottom panel) after 3 h centrifugation at 45,000*g*. The main peak of CCR activity was in the 27% (w/w) sucrose region of the gradients in the different buffers, whereas the position of MS activity peaks varied with the buffer used. MS cosedimented with CCR, in Tricine buffer only.

Evidence that MS is a peripheral membrane protein relies on data showing its solubilization in various salt solutions (3, 5, 13, 17, 25, 27) and its comigration with glyoxysomal membrane 'ghosts' in sucrose gradients using low ionic strength conditions (13, 14). Our results with cottonseed MS (Table I) were consistent with these experimental results, *i.e.* MS cosedimented with membranes in low ionic strength conditions but not in high ionic strength conditions. To reconcile our immunocytochemical and solubilization results, we hypothesized that MS was aggregating and sedimenting in membrane pellets under low ionic strength conditions. Addition of detergents to glyoxysomes isolated in low ionic strength solutions did not release a substantial percentage of MS which was consistent with aggregation phenomena. In maize glyoxysomes, MS was completely released in both low ionic strength and Triton X-100 solutions (22), indicating it was a matrix protein that did not exhibit aggregation behavior under these conditions. In our experiments, it was possible that the MS recovered in 100,000*g* pellets was sedimenting in association with detergent micelles. To address this possibility, we exploited the unique properties of Triton X-114 which undergoes phase separation at room temperature after extraction of lipophilic components in the cold. Large micelles can then be sedimented by low speed centrifugation (1,890*g*). We found that nearly all the MS from the aqueous phase was recovered in the 100,000*g* pellet in the absence of membrane components. Rate-zonal gradients (Fig. 2) indicated that MS in membrane pellets was highly aggregated, whereas MS in supernatants was not. Collectively, biochemical and immu-

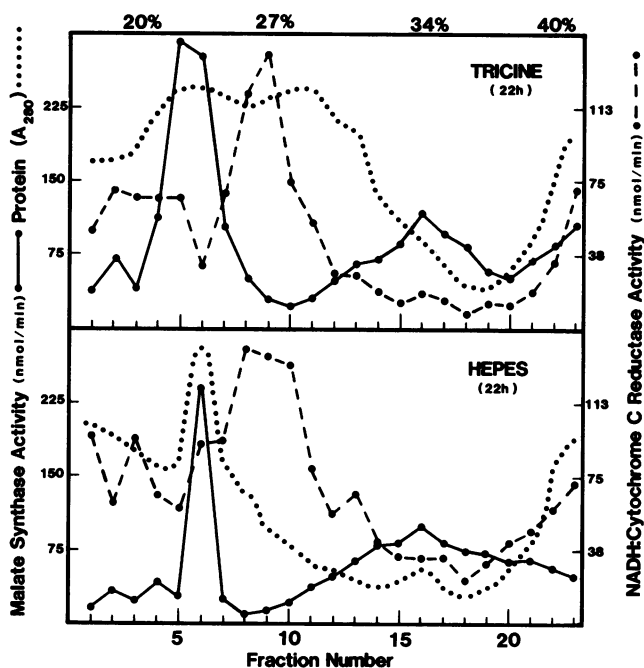

Figure 4. Centrifugation behavior of MS, CCR, and protein in sucrose gradients (percent w/w given at top), in 100 mM Tricine (pH 7.5) (top panel) and 10 mM Hepes (pH 7.0) (bottom panel) after 22 h centrifugation at 45,000*g*. The position of the main peak of CCR activity was in the 27% w/w region of both gradients. The positions of the two main MS activity peaks were similar in the two gradients, notably not coincident with the CCR peak.

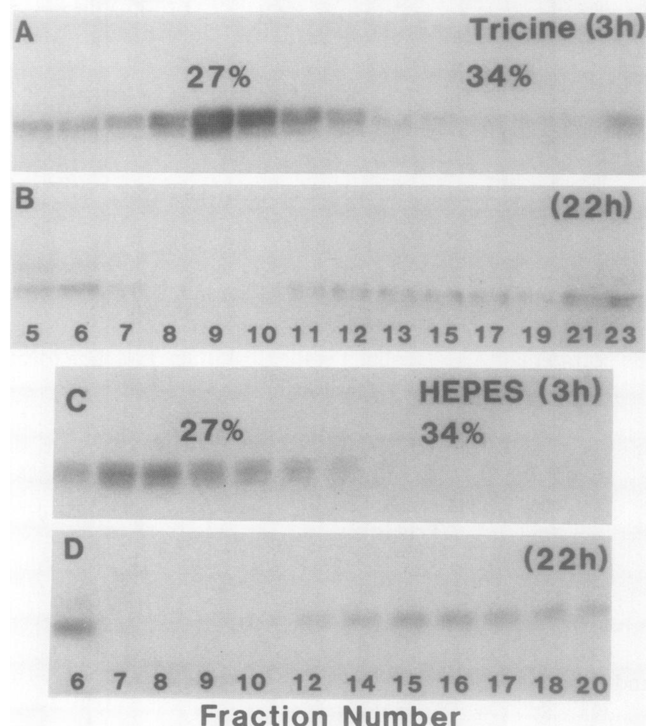

Figure 5. Western blots of sucrose-gradient fractions probed with affinity purified anti-MS IgGs. From Tricine-buffered gradients (A) after 3 h centrifugation (Fig. 3, top panel) and (B) after 22 h centrifugation (Fig. 4, top panel). From Hepes-buffered gradients (C) after 3 h centrifugation (Fig. 3, middle panel) and (D) after 22 h centrifugation (Fig. 4, lower panel). A constant amount of gradient sample (50 μL) was applied to each lane.

nocytochemical data showed that MS was a matrix protein, aggregated in relatively low ionic strength solutions, and disaggregated (or prevented from aggregating) in higher ionic strength solutions. We believe that aggregation behavior of MS has contributed to misinterpretations of its suborganellar location in glyoxysomes.

Interpretations as to whether MS was localized within the ER lumen (reflecting the site of synthesis) or cosedimented with ER vesicles in sucrose gradients (fortuitous association) were also most likely influenced by the aggregation behavior of MS. MS associated with ER in Tris- or Tricine-buffered sucrose-density gradients has been shown to be aggregated (4, 10, 18, 20). Gonzalez (10) reported that aggregated MS from castor bean endosperm sedimented with ER even after an extended centrifugation time (19 h), and when in the presence of $MgCl_2$ (magnesium shift). Because only a small region of the gradient enzyme activity profiles were presented in the paper (10), we cannot make comparisons to our contrasting gradient results (Figs. 3 and 4). Using cucumber cotyledons, Köller and Kindl (18) also found that MS sedimented independently from ER after extended centrifugation. In addition, they separated MS from ER vesicles in floatation experiments; MS continued to migrate to a higher density, while ER floated to its equilibrium density. They concluded that the MS peak represented a 'pro-glyoxysome' population of organelles which was an intermediate in their postulated scheme of glyoxysome biogenesis. In our experiments, K-phosphate eliminated MS aggregates in ER isolation gradients, *i.e.* MS activity (or protein, data not shown) did not appear in the ER region (Fig. 3, bottom panel). We believe that MS in the ER region of sucrose gradients is aggregated MS released from glyoxysomes during homogenization.

Table III. *Percent Enzyme Activity Remaining after Incubation of ER Fractions with Proteases*

Fractions containing peak CCR activity were either not treated (left in 27% sucrose), mixed with Triton X-100, or diluted in buffer to burst vesicles before being subjected to a mixture of proteolytic enzymes for 1 h at 37°C. Activities of MS, CCR, and FCR were measured before and after protease digestion and compared with control activities where the proteases were omitted.

Treatment	Enzymes		
	MS	CCR	FCR
		%	
27% sucrose, no Triton	2	14	100
27% sucrose + 0.5% Triton	2	15	100
No sucrose, no triton	5	ND	52

It was also reported that the MS in the ER region of castor bean gradients was protected from proteolytic cleavage by trypsin, which would indicate that MS was localized within ER cisternae (11). Contrary results were reported by Köller and Kindl (19) who separated the 100S aggregate in cucumber ER fractions from the ER vesicles by affinity chromatography. When our ER fractions were subjected to proteolytic diges-

tion, MS was completely digested in all treatments (Table III). Gonzalez (11) reported that approximately 50% of the MS activity after 1 h digestion of ER fractions and only about a 10% difference between treatments with and without 0.5% Triton X-100. In our work, FCR activity was not diminished except after vesicles were osmotically burst, indicating that vesicles were isolated intact and that the FCR catalytic site was oriented toward the lumen of the vesicles. On the other hand, about 85% CCR was digested with and without Triton X-100 (in sucrose), implying that this membrane protein's catalytic site was on the cytoplasmic surface of ER vesicles. Luster and Donaldson (24) have proposed a similar orientation for FCR and CCR in glyoxysomal membranes based on the same type of experimental results. Our data do not support the notion that MS is localized in the ER lumen, and therefore do not support the involvement of ER in MS biogenesis.

It seems clear from our data and results of others that MS is aggregated under certain preparative conditions. Reports where MS was purified from glyoxysomal membrane pellets probably were purifications of aggregated enzyme, not a peripheral membrane protein. Preparation of cell homogenates or organelles in K-phosphate (greater than 20 mM) allows one to work with MS that is not aggregated. The aggregation behavior of MS likely is not unique; putative glyoxysomal peripheral membrane proteins such as citrate synthase (28) and glycolate oxidase (15) exhibit similar sedimentation and solubilization characteristics. Studies similar to those reported herein on these and other enzymes most likely would generate an alternate concept of glyoxysomal enzyme intraorganellar organization.

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