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Regulation of Glyoxysomal Enzymes during Germination of Cucumber

3. *IN VITRO* TRANSLATION AND CHARACTERIZATION OF FOUR GLYOXYSOMAL ENZYMES[1]

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ABSTRACT

Monospecific antibodies raised against four glyoxysomal enzymes (isocitrate lyase, catalase, malate synthase, and malate dehydrogenase) have been used to detect these proteins among the products of *in vitro* translation in a wheat germ system programmed with cotyledonary RNA from cucumber seedlings. *In vitro* immunoprecipitates were compared electrophoretically with the same enzymes labeled *in vivo* and also with the purified proteins. Isocitrate lyase yields two bands on sodium dodecyl sulfate-polyacrylamide gels, as synthesized both *in vitro* (61.5K and 60K products) and *in vivo* (63K and 61.5K polypeptides). Both the 63K and 61.5K subunits can also be demonstrated for the isolated enzyme. The two subunits are antigenically cross-reactive and yield similar electrophoretic profiles upon partial proteolytic digestion. A larger subunit is seen *in vitro* than *in vivo* for both malate dehydrogenase (38K *versus* 33K) and catalase (55K *versus* 54K); this suggests a need for processing which is often a characteristic of proteins that must be transported across or into membranes. Malate synthase has a molecular weight of 57K both *in vitro* and *in vivo*, but the isolated enzyme is a glycoprotein, containing *N*-acetyl glucosamine, mannose, and possibly also fucose and xylose. This indicates that the polypeptide portion of the isolated enzyme is smaller than the *in vitro* product and suggests processing of malate synthase also. None of the other three enzymes appears to be glycosylated. The implications of these size differences for the compartmentalization of matrix and membrane-bound glyoxysomal enzymes are discussed.

Seed germination in fat-storing species such as cucumber requires a functional glyoxylate cycle to effect net gluconeogenesis from storage triglycerides (3). During early germination, glyoxylate cycle enzymes such as ICL[4] (*threo*-D-isocitrate glyoxylate lyase, EC 4.1.3.1) and MS (L-malate glyoxylate lyase, CoA-acetylating, EC 4.1.3.2) undergo a well characterized increase and subsequent decline in activity, with peak activity corresponding to the period of maximum fat metabolism (2, 3, 16, 32). We are interested both in the regulation of the appearance of glyoxylate cycle enzymes and in their compartmentalization within glyoxysomes (2). Crucial to such studies is the availability of purified glyoxysomal enzymes and of monospecific antibodies to them. We have reported previously (21) the isolation and immunological detection of ICL and catalase (H_2O_2/H_2O_2 oxidoreductase, EC 1.11.1.6). Here, we report the purification of MS from cucumber cotyledons and the immunological detection of both MS and glyoxysomal malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37).

Availability of these enzymes and their antibodies allows the detection of the relevant polypeptides among the products of *in vitro* translation in a cell-free system programmed with cotyledonary RNA from different stages in germination. Our analysis of the messenger RNAs for these enzymes and the temporal relationship of their appearance to the enzyme activity profiles will be described in a future publication.

To understand how these proteins are packaged into glyoxysomes, it is desirable to know whether they are synthesized in precursor form and, if so, how they are modified during or after translation. It is, therefore, of interest to determine whether these proteins carry a "signal" sequence of possible significance in compartmentalization (4). We have used SDS-PAGE to compare *in vitro* translation products with glyoxysomal proteins labeled *in vivo*. To determine whether these enzymes have passed through the ER, we have also assayed for glycosylation by several techniques, since the process of *N*-glycosylation has been localized to the ER in plants (26). In addition, we have established that cucumber ICL exists in two forms, with subunits that differ in mol wt, but are antigenically cross-reactive and yield very similar products upon limited proteolysis in SDS gels. Several aspects of this work have already been reported in preliminary form (28, 29).

MATERIALS AND METHODS

Sources. All materials were obtained as previously indicated (21), except for the following: acetonitrile (spectrophotometric grade) from Aldrich, proteinase K from Beckman, *Staphylococcus aureus* V-8 protease from Miles, and cyanogen bromide, Sepharose 4B, CM-Sephadex, and Triton X-100 from Sigma. [^{35}S]Methionine (translation grade, 500–1,000 Ci/mmol) and Na^{125}I (carrier-free) were products of New England Nuclear. Reagents for gas chromatography were spectrophotometric grade from Mallinckrodt. *S. aureus* Cowan I was a generous gift from Dr. Richard Meagher at the University of Georgia and the methicillin-resistant strain A676

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[4] Abbreviations: ICL: isocitrate lyase; MS: malate synthase; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CAT: catalase; HTP: hydroxylapatite; MDH: malate dehydrogenase; buffer A: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NaN_3; IgG; immunoglobulin G; TNT: 100 mM Tris-HCl (pH 8.6), 150 mM NaCl, 1% Triton X-100; ConA: concanavalin A.

of the same organism from Dr. Göran Kronvall at the University of Lund, Sweden. Human IgG was kindly provided by the Blood Transfusion Service, Protein Fractionation Centre, Edinburgh, Scotland.

Isolation of Enzymes. CAT and ICL were purified as described previously (21) except that for ICL, Sepharose 6B was replaced with Ultra-Gel AC-34 (2.5 × 80 cm column) and the DEAE-cellulose flow-through and gradient ICL peaks were pooled separately for chromatography on Bio-Gel-HTP as described (21). MS was purified by a modification of the procedure of Köller and Kindl (20). Cotyledons from 3-day dark-grown cucumbers were homogenized in 3 volumes of 50 mM Tricine-KOH (pH 7.7), 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, and centrifuged at 9,000*g* for 3 min. The resulting supernatant was centrifuged at 40,000*g* for 2 h, the pellet washed in grinding buffer and recovered by centrifugation. The pellet was resuspended in a small volume of 50 mM Tris-HCl (pH 8.5) to which 0.2 volumes of 600 mM $MgCl_2$, 12 mM sodium glyoxylate was then added. The slurry was stirred for 30 min at 4 C and centrifuged at 85,000*g* for 2 h. The MS-containing supernatant was chromatographed on Sepharose 6B as described by Köller and Kindl (20). The MS peak fractions were concentrated by precipitation with $(NH_4)_2SO_4$ (80% saturation), and dissolved in 10 mM Hepes (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM sodium glyoxylate, dialyzed against the same buffer, and chromatographed on CM-Sephadex (20). The MS peak fractions were pooled, used for antiserum production, and analyzed by SDS-PAGE (21). The final specific activity of the purified MS was 20,000 to 25,000 units/mg. Glyoxysomal MDH from watermelon was generously provided by Dr. B. Hock at the University of Bochum.

Preparation of Antisera. Our procedures for the preparation of antisera in both rabbits and mice have been described (21). All antibodies were fractionated by $(NH_4)_2SO_4$ precipitation (21) and in some cases further purified on protein A-Sepharose 4B.

Glyoxysome Preparation. Glyoxysomes were prepared from cucumber cotyledons by differential centrifugation, followed by equilibrium density centrifugation in sucrose. Cotyledons from 3-day dark-grown cotyledons were chopped with razor blades, then ground in a mortar and pestle in 2 volumes of grinding medium (50 mM Tricine-KOH [pH 7.7], 4 mM $MgCl_2$, 1 mM EDTA). The homogenate was filtered through cheesecloth and centrifuged at 9,000*g* for 3.5 min. The supernatant was then layered over a linear sucrose gradient (40–60% [w/w] sucrose in grinding medium) and centrifuged at 58,000$g_{average}$ for 3 h (Spinco SW 25.2 rotor). After centrifugation the tube was punctured and 1.3-ml fractions were collected from the bottom. The three peak ICL-containing fractions were pooled as glyoxysomes.

Preparation of Human IgG-Sepharose 4B. Activated Sepharose 4B beads were prepared by the procedure of March *et al.* (24). To 47 ml of activated beads were added 77 ml of human IgG (15 mg/ml) in 0.2 M $NaHCO_3$ (pH 9.5). The beads were shaken at 4 C for 20 h and collected by filtration. The filtrate was analyzed for protein by the method of Kalb and Bernlohr (17); coupling was estimated at 92%. The beads were resuspended in 1 M ethanolamine (pH 9.0) and shaken at room temperature for 2 h to mask any unreacted groups. The beads were then washed as described by March *et al.* (24), packed into a glass column (2.0 cm i.d.), and washed extensively with buffer A, then with 0.1 M glycine (pH 3.0), and finally with buffer A again.

Culture of *S. aureus*. *S. aureus* Cowan I was grown and fixed as described by Kessler (18) except that no vitamins were added to the culture medium. The methicillin-resistant strain A676 of *S. aureus* was also cultured according to Kessler (18), again without addition of vitamins.

Preparation of Protein A. Protein A (an immunoglobulin-binding protein) was purified from culture filtrates of the methicillin-resistant strain A676 of *S. aureus*. After 18 h of growth the cells were pelleted by centrifugation at 10,000*g* for 10 min and the supernatant was applied directly to a column of human IgG-Sepharose 4B and washed extensively with buffer A. Protein A was then eluted with 0.1 M glycine (pH 3.0), neutralized with 1 M Tris-HCl (pH 8.8), and precipitated with 80% saturated $(NH_4)_2SO_4$. The pellet was dissolved in H_2O and dialyzed extensively, then analyzed by UV scanning (31), SDS-PAGE (21), and immunoelectrophoresis (21). The protein A was homogeneous by all three criteria.

Preparation of Protein A-Sepharose. Protein A-Sepharose 4B was prepared as described for human IgG-Sepharose 4B above, except that 4 ml of a solution of 4 mg/ml protein A was used with 8 ml of activated Sepharose 4B. Virtually all of the protein A was coupled by this procedure, as quantitated spectrophotometrically.

Preparation of IgG. Antisera or $(NH_4)_2SO_4$-precipitated antibodies were applied to protein A-Sepharose 4B. The column was washed and eluted as in the purification of protein A above. The protein content of the purified IgG was estimated using the relationship 1.4 A_{280} units of IgG = 1 mg (27). IgG preparations were stored at −20 C. This preparative procedure inactivated CAT antibody.

***In Vitro* Protein Synthesis.** Protein synthesis was carried out *in vitro* using a cell-free wheat germ system programmed with total cotyledonary RNA isolated from 3-day dark-grown cotyledons. The reaction was allowed to proceed at 25 C for 60 min, after which polyribosomes were removed by centrifugation (300,000g_{max} for 1 h) and the supernatant was assayed for relevant polypeptides by immunoprecipitation.

Immunoprecipitation. Extracts were prepared for immunoprecipitation by adding an equal volume of 2× TNT buffer and 7 μg of IgG, followed by incubation of the extract at 37 C for 30 min. Then fixed *S. aureus* Cowan I cells (20 μl of a 10% suspension, prewashed in TNT buffer) were added and incubation was continued for 10 min at room temperature. The extract was layered over 15% (w/v) sucrose in TNT buffer and the cells were collected by centrifugation at 5,000*g* for 5 min. The cells were washed three times in TNT buffer containing 0.1% SDS and once in 0.12 M Tris-HCl (pH 6.8). The pellet was resuspended in 50 μl of 2× electrophoresis sample buffer (21), and boiled for 2 min. The cells were then pelleted at 10,000*g* for 5 min and the supernatant was analyzed by SDS-gel electrophoresis.

***In Vivo* Labeling.** Thirty 2-day dark-grown cotyledons were excised, surface-sterilized for 5 min in 70% ethanol, then for 5 min in 1% NaCl followed by five rinses in sterile distilled H_2O. The cotyledons were incubated upright for 48 h in the dark in 0.5 ml of sterile distilled H_2O containing 0.5 mCi [^{35}S]methionine. Sterile distilled H_2O was added as necessary to maintain volume. Cotyledons were then rinsed and homogenized in 0.1 M Tris HCl (pH 8.6) 0.2 M NaCl, 10 mM $MgCl_2$. Following centrifugation at 286,000*g* for 1 h (Spinco SW 41 rotor), the supernatant was used for immunoprecipitation. Alternatively, after labeling in the same manner, glyoxysomes were isolated as described above, except that the procedure was scaled down and the gradients were run in the Spinco SW 41 rotor; fraction size was 0.5 ml instead of 1.3 ml. The three peak ICL-containing fractions were pooled and used for immunoprecipitation.

Limited Proteolysis of ICL-A and ICL-B Subunits. ICL was resolved into forms A and B on 7.5% SDS-polyacrylamide gels (10 × 200 × 1.5 mm) and electrophoresed with protease as described by Cleveland *et al.* (10) except that a stacking gel of only 1.5 cm was used on the digesting gel. Proteases and concentrations used are indicated in the legend to Figure 5.

Sugar Analysis by Gas Chromatography. About 1 mg of purified protein was precipitated with 10% cold trichloroacetic acid for 30 min at 0 C. The pellet was washed once with cold 5% trichloroacetic acid and five times with 80% (v/v) acetone before a final wash with 100% acetone. The enzyme was digested for 2 h at 121 C under N_2 in 2 N trifluoroacetic acid, reduced, acetylated, and analyzed on a Hewlett Packard 5830A gas chromatograph as

described by Burke and Keegstra (7). A standard containing 1.0 mg of BSA and 10 μg each of glucose, mannose, galactose, and glucosamine was run in parallel.

^{125}I-Con A-binding Assay. Con A was purified by the procedure of Agrawal and Goldstein (1). The iodination, repurification, and binding assay were performed as described by Burridge (9).

RESULTS

MS Purification. MS was purified from total cellular membranes of cucumber cotyledons using a modification of the procedure described previously by Köller and Kindl for MS isolation from glyoxysomal membranes (20). The specific activity of the purified enzyme was 20,000 to 25,000 units/mg, which compares well with that reported by Köller and Kindl. The enzyme appears to be homogeneous, since a single band is seen upon SDS-PAGE. The MS subunit migrates slower than the catalase subunit (54,000), but faster than that of ICL (63,000), with an estimated mol wt of 57,000 on 15% gels (Fig. 1). MS also runs as a polypeptide of 57,000 daltons on 10 and 12% gels (data not shown). This is a lower mol wt than the values of 63,000 to 64,000 reported by others (6, 20). It is unlikely that the value of 57,000 is an artifact due to enzyme modification during the isolation procedure, since purified MS co-migrates upon SDS-PAGE with the major protein band obtained by salt elution of glyoxysomal membranes, an early step in the procedure used for MS isolation by Köller and Kindl (20).

The antigenic purity of isolated MS is shown in the immunoelectrophoretogram of Figure 2. Admittedly, however, this procedure is not likely to detect trace contaminants in the enzyme preparation, since the rabbit used in antiserum production received only 25 μg of protein/kg body weight at each injection. The MS precipitation line is long, consistent with the existence of two forms of MS, one small and acidic, the other large and basic (20). The small acidic form has presumably migrated a significant distance toward the anode, while the large basic form moved only slightly at this pH, forming two precipitation arcs which join, showing complete antigenic identity.

Multiple Forms of ICL. Since publication of our initial report on ICL purification (21), it has become apparent that cucumber ICL exists in at least two separable forms. When chromatographed on DEAE-cellulose, ICL activity is recovered both in the flow-through and in the gradient (21). Upon further purification of these two activities on hydroxylapatite, the enzyme can be resolved into two forms, ICL-A and ICL-B, differing in subunit mobility upon SDS-PAGE as shown in lanes a and b of Figure 3. Mol wt at 63,000 for ICL-A (lane a) and 61,500 for ICL-B (lane b). ICL-B is probably not an artifact of ICL-A degradation during purification, as both subunits are found in isolated glyoxysomes (Fig. 4), and the relative rates of ICL-A and ICL-B accumulation appear to be regulated developmentally (D. E. Titus and H. Riezman, unpublished data). Neither form appears to be glycosylated (see below). Sequence similarity between the two forms of ICL is suggested both by comparison of the products of limited proteolytic digestion (Fig. 5) and by antigenic cross-reactivity (*i.e.* antibodies raised against form A react specifically with both A and B). Two forms of ICL have also been reported for flax seedlings (19), but no determination of subunit mol wt was mentioned.

***In Vitro* Translation.** Availability of antibodies against ICL, CAT, MS, and MDH allows the detection of these enzymes among the products of *in vitro* translation as well as in cotyledonary extracts from seedlings labeled *in vivo*. Figure 3 illustrates the results obtained when a wheat germ system is programmed with total cotyledonary RNA from 3-day dark-grown seedlings and the products are then analyzed by immunoprecipitation, SDS-PAGE,

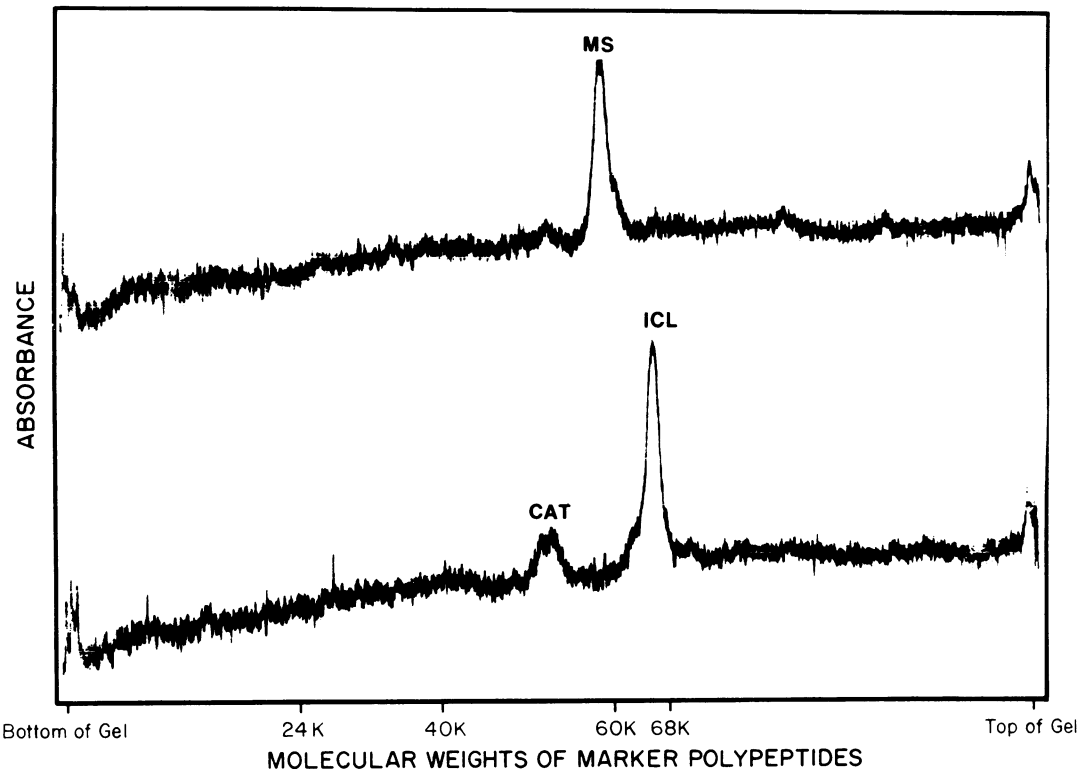

FIG. 1. Densitometric scan of purified MS. Purified MS (upper scan) and a fraction from DEAE-cellulose containing both ICL and CAT (lower scan) were electrophoresed on a 15% SDS-polyacrylamide slab gel. The gel was stained with Coomassie blue, destained, and dried onto Whatman 3MM paper. The gel was photographed using a Polaroid MP-4 Land Camera and Type 55 Land film (4 in x 5 in). The negative was fixed, washed and dried, then scanned using a Joyce Loebl & Co microdensitometer.

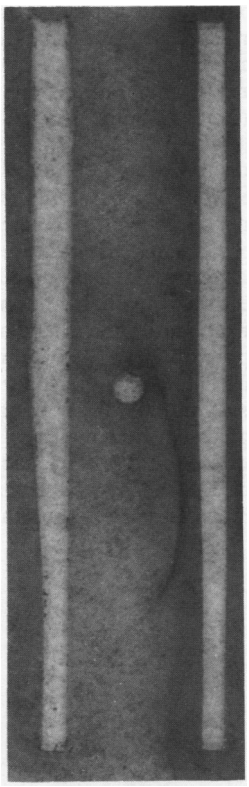

FIG. 2. Immunoelectrophoresis of MS. A crude homogenate from 3-day dark-grown cotyledons was concentrated by precipitation with 80% acetone, washed once in 80% acetone, and resuspended in 0.04 M Na-barbital (pH 8.6). The sample was then loaded into the center well and electrophoresed at 5 mamp for 1 h. The troughs were removed and antiserum against MS was placed in the right-hand trough, with control serum in the trough on the left. Immunodiffusion was allowed to proceed for 24 h at room temperature. The plate was then washed 1 day in 0.15 M NaCl and for 2 days in H_2O. The agarose was pressed, stained with Coomassie blue, and destained.

and autoradiography. All four glyoxysomal enzymes are synthesized *in vitro* in readily detectable quantities, as shown by the bands in Figure 3 specifically precipitated by antibodies against ICL (lane c), CAT (lane g), MS (lane k), and MDH (lane o). In each case, the *in vitro* product is compared directly with the same enzyme synthesized and labeled *in vivo* (lanes d, f, l, and n) and with the purified protein (lanes a and b, h, j, and p). Shown in addition are profiles of glyoxysomal matrix proteins (lane e; note the prominence of ICL-A, ICL-B and CAT) and salt-eluted peripheral membrane proteins (lane m; note the prominence of MS).

Two immunoprecipitable ICL bands are routinely detectable among the products of *in vitro* translation (lane c), although with variable relative intensities. These have estimated mol wt of 61,500 and 60,000, whereas the protein as labeled *in vivo* yields bands at approximately 63,000 and 61,500 (lane d), corresponding to the subunits of ICL-A and ICL-B (lanes a and b), respectively. Bands of these latter mol wt are also prominent in the glyoxysomal matrix fraction (lane e), and can be immunoprecipitated from isolated glyoxysomes. It is not yet clear what relationship exists between the two ICL polypeptides synthesized *in vitro* and the two slightly larger subunits found *in vivo*. Two-dimensional gel electrophoresis and limited proteolytic digestion should be useful in establishing the correspondence between the *in vitro* and *in vivo* bands; such experiments are in progress.

For CAT, the single immunoprecipitable product from *in vitro* synthesis (lane g) has a mol wt of about 55,000, approximately 1,000 daltons larger than either the *in vivo* product (lane f) or the isolated enzyme (lane h). The MDH subunit synthesized *in vitro* (lane o) is also larger then either the *in vivo* product (lane n) or the isolated enzyme (lane p), but in this case the difference is about 5,000 daltons (38,000 *in vitro versus* 33,000 *in vivo*), in good agreement with the findings of Walk and Hock (33). We do not, however, detect any evidence of *in vitro* processing, as reported by these authors. For MS, both the *in vitro* and the *in vivo* translation products yield a single immunoprecipitable polypeptide of 57,000, identical in size to that of the isolated enzyme.

MS Is a Glycoprotein, ICL is Not. Because of the known effect of sugar residues on polypeptide mobility in SDS gels, ICL, MS, CAT, and MDH were assayed for sugar content, using both lectin binding and gas chromatographic analysis. ^{125}I-Con A showed no detectable binding to either CAT or MDH (data not shown). Con A appears to bind to ICL (Fig. 6), but the binding is not reversible with hapten (α-methylglucoside) and is therefore nonspecific, possibly involving the hydrophobic site of Con A (13). With MS, however, Con A binding is specific and hapten-reversible (Fig. 6). This distinction between MS and ICL is confirmed by the sugar analysis presented in Table I. MS is clearly a glycoprotein, containing *N*-acetyl glucosamine, mannose, and possible fucose and xylose, but no sugars can be detected with either ICL-A or ICL-B, down to a limiting resolution of 0.3 mol sugar/mol polypeptide. We therefore conclude that ICL is not a glycoprotein, contrary to the claim of Frevert and Kindl (12). In fact, their findings appear to support the same conclusion, since we calculate from their data that no more than 0.1% of the enzyme molecules were labeled upon cleavage with periodic acid and reduction with NaB^3H_4. These workers (12) also used [^{3}H]glucosamine incorporation as evidence of ICL glycosylation, but no evidence was provided to establish that the label incorporated into ICL was still in the form of glucosamine.

Both Con A binding and direct assay establish MS as a glycoprotein. This confirms the report of Mellor *et al.* (25) based on periodic acid-Schiff staining. Since the enzyme is synthesized *in vitro* with a mol wt that is indistinguishable from the isolated enzyme (Fig. 3) and the *in vitro* wheat germ system does not

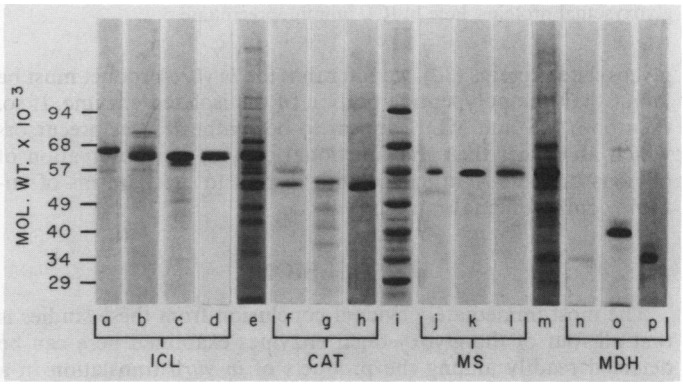

FIG. 3. *In vitro* and *in vivo* synthesis of glyoxysomal enzymes. Proteins synthesized *in vitro* and *in vivo* in the presence of [^{35}S]methionine were immunoprecipitated with monospecific antibodies as described in the text and electrophoresed on 10% SDS-polyacrylamide gel. The gels were prepared for fluorography (5), then stained with Coomassie blue for 5 min and destained for 1 h. The gels were dried and exposed to Kodak No Screen Medical x-ray film at −70 C. Lanes c, d, f, g, k, l, n, and o were excised from such autoradiograms and therefore depict ^{35}S-labeled polypeptides. Lanes a, b, e, h, i, m, and p are strips from stained gels, showing stained, unlabeled polypeptides. a: Isolated ICL-A; b: isolated ICL-B; c: ICL *in vitro*; d: ICL *in vivo*; e: glyoxysomal matrix proteins; f: CAT *in vivo*; g: CAT *in vitro*; h: isolated CAT; i: mol wt markers; j: isolated MS; k: MS *in vitro*; l: MS *in vivo*; m: glyoxysomal peripheral membrane proteins; n: MDH *in vivo*; o: MDH *in vitro*; p: isolated MDH.

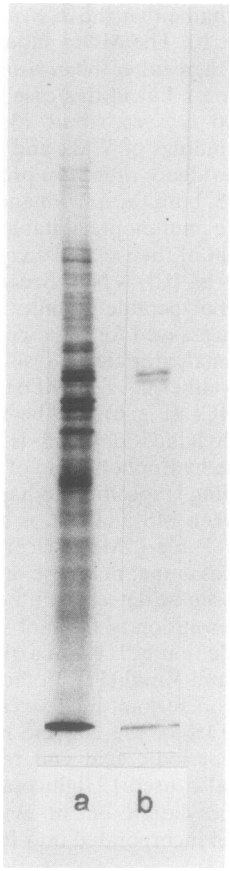

FIG. 4. Cotyledons were labeled with [^{35}S]methionine, and glyoxysomes were prepared as described in the text. ICL was immunoprecipitated from these glyoxysomes using antibody raised against purified ICL-A. The immunoprecipitate and the glyoxysomal preparation were then run on a 10% SDS-polyacrylamide gel which was stained, destained, dried, and exposed to Kodak No Screen Medical x-ray Film for 5 days. Lane a: glyoxysomal proteins; lane b: ICL immunoprecipitate.

glycosylate proteins (30), we infer that the *in vitro* product must be larger than the polypeptide portion of the isolated enzyme. If so, then both MS and MDH appear to be synthesized as precursors which are larger than the functional enzyme, an observation of obvious possible significance with respect to mechanisms of enzyme compartmentalization.

DISCUSSION

The most immediately evident conclusion from these studies is that all four of the glyoxysomal enzymes examined here can be detected readily among the products of *in vitro* translation in a cell-free system programmed with cotyledonary RNA, with specific, unequivocal indentification provided by immunoprecipitation, electrophoresis, and autoradiography. This makes it possible to assay for translatable mRNA during the course of germination and seedling emergence and thereby to determine the relationship between the appearance of glyoxylate cycle enzyme activities and the functional availability of individual mRNAs. The results of such a developmental analysis will be described in a forthcoming paper.

The ability to synthesize specific glyoxysomal enzymes *in vitro* also facilitates investigation of the mechanisms of enzyme compartmentalization and organelle biogenesis. Two somewhat different models for microbody biogenesis have been suggested recently. Goodman and Blobel (14) proposed that microbody matrix enzymes are synthesized on free ribosomes and are transported posttranslationally into a premicrobody structure, while proteins destined for association with the microbody membrane are synthesized on membrane-bound polysomes and arrive at the microbody membrane by membrane flow. On the other hand, Lord and Bowden (22) postulated that all of the enzymes are synthesized on membrane-bound polysomes, segregated into cisternae of the ER, and subsequently packaged into glyoxysomes of the latter bud off the ER. The availability of ICL, CAT, MS, and MDH and antiserum against each facilitates the testing of these models, since the former two enzymes are glyoxysomal matrix enzymes, while the latter two are both membrane-bound (20).

In animal cells, vectorial transport of secretory proteins across the ER occurs co-translationally and is mediated by a hydrophobic signal sequence of approximately 2,000 daltons at the *N*-terminus (11). Such proteins are detected in precursor form *in vitro* because cell-free translation systems lack the peptidase required for processing, A possible signal sequence has been reported for zein, the storage protein sequestered into protein bodies in corn (8).

Our results indicate that MDH and perhaps also MS are synthesized as a larger polypeptide *in vitro* than *in vivo*. For MDH,

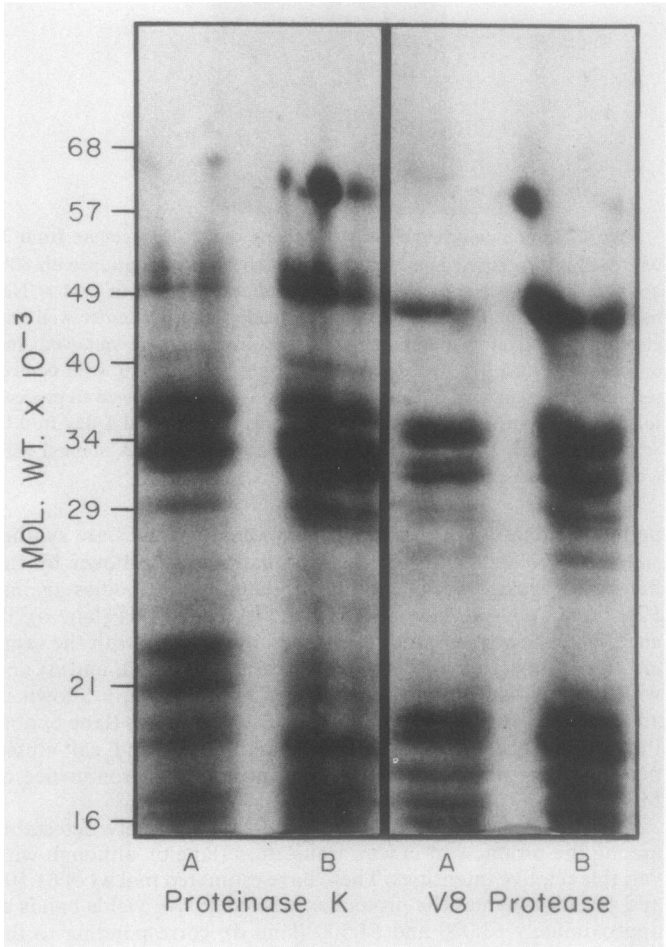

FIG. 5. Limited proteolysis of ICL-A and ICL-B. Purified subunits A (63,000 daltons) and B (61,500) of ICL were resolved on 7.5% SDS-polyacrylamide gels. The gel slices were prepared according to Cleveland *et al.* (10) and approximately 20 μg of each subunit was loaded into slots of a second SDS-polyacrylamide gel (15%). The gel slices were overlaid with 40% sucrose, then 20% sucrose containing either proteinase K or *S. aureus* V-8 protease (0.05 and 0.2 μg, respectively). The samples were allowed to stack, then the current was turned off for 10 min. Thereafter, the current was turned on again and electrophoresis continued until the tracking dye (bromophenol blue) reached the bottom of the gel. The second dimension separating gel was 0.75 mm thick.

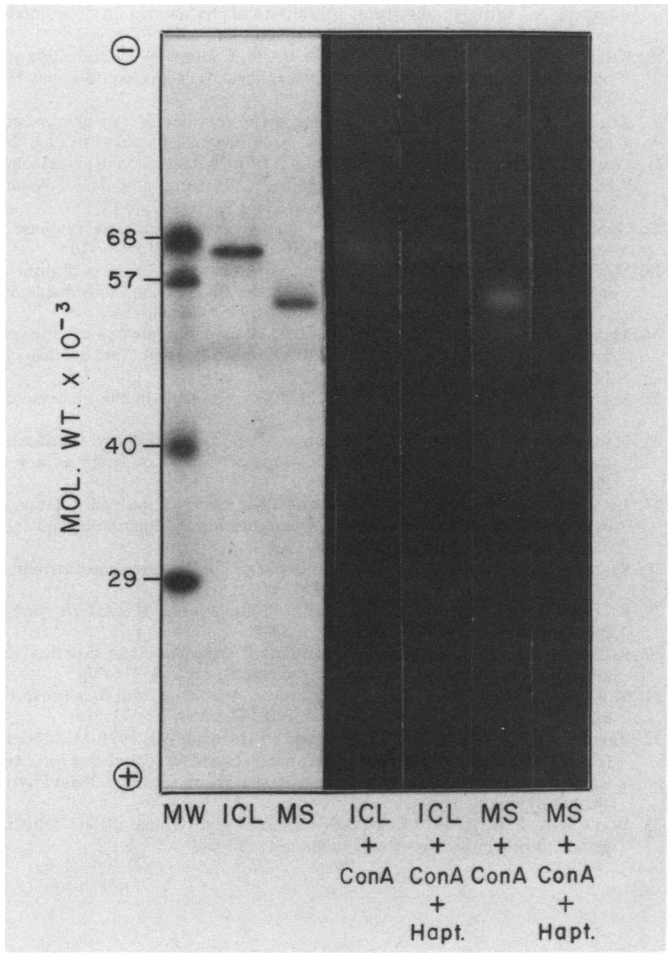

FIG. 6. Con A labeling of MS. ICL and MS were electrophoresed on 15% SDS-polyacrylamide gels, stained with Coomassie blue, and destained. The gels were equilibrated and "stained" with ^{125}I-Con A (+ Con A) in the presence (+ Hapt) or absence of 30 mg/ml α-methylglucoside, as described by Burridge (9).

the difference is about 5,000 daltons, in good accord with the finding of Walk and Hock (33). This is larger than expected for a signal sequence, but is not quite as large as the difference reported between the *in vitro* and *in vivo* products for the small subunit of ribulose-1,5-bisphosphate carboxylase, which is thought to be synthesized on cytoplasmic ribosomes and then transported into the chloroplast posttranslationally (15).

At first glance, there appears to be no evidence for a signal sequence in the case of MS, since the *in vitro* product has the same mol wt as the isolated enzyme. However, the functional enzyme is most likely a glycoprotein, and the *in vitro* wheat germ system does not glycosylate proteins (30). Since carbohydrates usually decrease the mobility of proteins in SDS gels, it is tempting to speculate that the polypeptide portion of MS would actually have a mol wt less than 57,000 and the nonglycosylated, *in vitro* product would be larger. Preliminary experiments using tunicamycin, an inhibitor of glycosylation *in vivo*, indicate that the polypeptide portion of MS is in fact smaller than the isolated enzyme (work in progress). Therefore, MS and MDH, both membrane-bound glyoxysomal enzymes, may be synthesized *in vitro* as a larger precursor.

A reproducible size difference between *in vitro* and *in vivo* products is also seen for catalase, a matrix protein. The difference is much smaller than that for MDH, and is consistent with a possible signal sequence. This is in contrast to rat liver catalase, which is reportedly synthesized on free polysomes and for which

Table I. *Sugar Analysis of MS and ICL*

MS and ICL were assayed for sugar content by gas chromatography as described in the text. Ovalbumin (OVAL) was included as a positive control, since it is a glycoprotein known to contain *N*-acetyl glucosamine and mannose. BSA is not glycosylated and was included as a negative control. Polypeptide mol wt assumed in calculating sugar content were: MS, 57,000; ICL, 63,000; OVAL, 45,000; and BSA, 68,000. GlcNAc = N-acetyl glucosamine; ND = not detected (less than 0.3 mol sugar/mol polypeptide).

Protein	Sugar Content			
	GlcNAc	Mannose	Fucose	Xylose
	mol/mol polypeptide			
MS	1.0	1.4	0.4	0.7
ICL	ND	ND	ND	ND
OVAL	2.9	3.7	ND	ND
BSA	ND	ND	ND	ND

the *in vitro* product is identical in size to the isolated enzyme (14). For ICL, analysis is complicated by the existence of two forms both *in vitro* and *in vivo* and by our current uncertainty concerning the relationship between the *in vitro* products and the two forms in which the enzyme can be isolated. Since a 61,500-dalton band is found both *in vitro* and *in vivo*, it may well be that ICL, a matrix protein, is not synthesized in precursor form. Alternatively, if the larger and smaller products *in vitro* actually give rise, respectively, to the larger and smaller subunits found *in vivo*, processing must involve a gain rather than a loss in mol wt. As we do not consider ICL to be a glycoprotein, some mechanism other than glycosylation must be postulated to explain the increase in mol wt. In this case, methylation and acetylation are possible.

Thus, several glyoxysomal enzymes appear to be synthesized in a precursor from that requires processing, but a clear dichotomy between matrix and membrane-bound proteins does not emerge from the data at present. The fact that MS seems to be a glycoprotein suggests that it is synthesized on membrane-bound polysomes, since glycosylation is thought to take place during translation on the ER (26). This lends support to both models of microbody biogenesis (14, 22), because MS is a membrane-bound enzyme. MDH and CAT are both synthesized as larger precursors *in vitro* and the size of the extra sequence is compatible with a signal sequence, at least in the latter case. This does not necessarily indicate that CAT is segregated into the ER during its synthesis, since an extra sequence of similar size is found on the β-subunit of F_1-ATPase, yet this polypeptide appears to be transported into the mitochondrion after translation (23). ICL is not synthesized as a larger precursor *in vitro*, which seems to support the model of Goodman and Blobel (14).

In experiments involving isolated membrane-bound and free polysomes, we have been able to detect immunologically all four enzymes among the *in vitro* translation products for both classes of polysomes (data not presented). We are, therefore, unable to confirm either of the two alternative models for microbody biogenesis as presently formulated. A detailed understanding of the mode of glyoxysome biogenesis and enzyme compartmentalization is likely to await definitive experiments in which the vectorial enzyme transport system is reconstructed *in vitro*, using membranes of stripped rough ER, "nascent" glyoxysomes, or the organelle itself.

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