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CELL-FREE SYNTHESIS OF GLYOXYSOMAL MALATE DEHYDROGENASE

R.-A. Walk and B. Hock

Lehrstuhl für Allgemeine Botanik, Arbeitsgruppe Biochemie der Morphogenese, Ruhr-Universität Bochum, Postfach 102148, D-4630 Bochum 1, Federal Republic of Germany

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SUMMARY

Polyadenylated mRNA was isolated from germinating watermelon cotyledons and translated in a wheat germ protein synthesizing system. The synthesis of glyoxysomal malate dehydrogenase was detected by direct immunoprecipitation and electrophoretic analysis of the precipitate. In addition to a small amount of the authentic isoenzyme (subunit molecular weight = 33 000), the major part of the incorporated [^{35}S] methionine was observed in a polypeptide with a molecular weight of 38 000. The possible role of the larger molecule as a precursor of glyoxysomal malate dehydrogenase is discussed.

INTRODUCTION

The biogenesis of cell organelles requires a highly specific distribution of proteins synthesized in the cytoplasm. This is typically reflected in the unique pattern for localization of isoenzymes in different cell organelles.

Cotyledons of dark-grown watermelon seedlings are known to contain one mitochondrial, one glyoxysomal and three cytosolic isoenzymes of malate dehydrogenase (MDH; EC 1.1.1.37) (1). The glyoxysomal isoenzyme is synthesized by cytoplasmic ribosomes (2) and is subsequently integrated into glyoxysomes.

Based upon electron microscopic observation of membrane continuities between rough endoplasmatic reticulum and glyoxysomes (3) and a proposed mechanism for synthesis of secretory proteins by membrane-bound polysomes (4), the hypothesis

Abbreviations: MDH: malate dehydrogenase
SDS-PAGE: sodium dodecylsulfate - polyacrylamide gel electrophoresis

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was put forward (2) that the distribution of isoenzymes might be determined by the presence or absence of a signal sequence in messenger RNA (mRNA), a sequence that is crucial for the binding of polysomes to intracellular membranes (4). According to this hypothesis, it was expected that in a cell-free protein-synthesizing system mRNA coding for glyoxysomal MDH should be translated into a product which contains a corresponding signal peptide and is larger than the subunit for authentic isoenzyme.

Therefore, we have isolated polyadenylated mRNA from watermelon cotyledons and translated it in a cell-free system derived from wheat germ. The newly synthesized products were analyzed with antibodies against glyoxysomal MDH and the molecular weight of the immunoprecipitated polypeptides was determined by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE).

MATERIALS AND METHODS

Watermelon seeds (Citrullus vulgaris Schrad.) were grown for two days in the dark as described previously (5). The isolation of mRNA was based upon the method of Aviv and Leder (6): 54 g of cotyledon tissue were homogenized in 200 ml 50 mM tricine, 120 mM NaCl, 20 mM EDTA, 2% SDS, pH 9.5, and 200 ml phenol-chloroform-isopentenyl alcohol (50:49:1, v/v/v) for 3 x 15 s with a VirTis homogenizer at 45 000 rpm. After separation of the aqueous from the organic phase by centrifugation (4 100 x g for 15 min at 1°C), the aqueous phase was reextracted four times with 1/2 volume of fresh phenol-chloroform-isopentenyl alcohol. The nucleid acids were precipitated with ethanol at -30°C and dissolved in 10 mM triethanolamine, 1 mM EDTA, 0.4% SDS, pH 7.5, and reprecipitated with ethanol. The pellet was washed twice with 3 M sodium acetate, pH 6.0 according to Palmiter (7). The washed precipitate was dissolved in 10 mM tricine, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS, pH 7.5, and finally chromatographed on oligo(dT)-cellulose (Collaborative Laboratories, USA) equilibrated with the same buffer. Bound RNA was eluted from the column with 10 mM tricine, 1 mM EDTA, 0.05% SDS (pH 7.5) at 50°C. This fraction of polyadenylated RNA was precipitated with ethanol, dissolved with water and stored at -80°C.

All materials which could come into contact with nucleic

Table 1

Radioactivity (cpm) of hot trichloroacetic acid insoluble material of aliquots (5 µl) removed from wheat germ incubation mixtures after 90 min at 25°C. Counting efficiency on Whatman 3MM filter discs suspended in 5 ml scintillator was 70-80%. The figures given for radioactivity are the average of two determinations whose difference was < 7%.

Sample	cpm
control (H_2O)	3 822
watermelon poly(A)$^+$-mRNA (6.6 µg)	180 740
watermelon poly(A)$^+$-mRNA (6.6 µg) + cycloheximide (15 µg/ml)	6 153
watermelon poly(A)$^+$-mRNA (6.6 µg) + D(+)threo chloramphenicol (400 µg/ml)	198 814
rat liver polysomal poly(A)$^+$-mRNA (3.6 µg)	172 742

acid fractions were rinsed with a hot detergent (1% Deconex) and autoclaved subsequently. Reagent solutions were boiled with 0.1% diethylpyrocarbonate and autoclaved.

Preparations of wheat germ extracts, cell-free protein synthesis and determination of the incorporation of radioactive [^{35}S]methionine (3.9×10^{13} $s^{-1} \cdot mmol^{-1}$, Amersham Buchler, UK) into proteins were performed as described by Roberts and Paterson (8). The wheat germ extract was used without preincubation.

Glyoxysomal MDH from cell-free mixtures was precipitated with monospecific antibodies (2) in the presence of purified carrier antigen (9) and the precipitates were washed according to Daniel et al. (10). Sample-preparation for electrophoretic analysis, SDS-PAGE and autoradiography were performed as described by Blobel and Dobberstein (4).

RESULTS

Polyadenylated mRNA has been extracted from cotyledons of 2 day germinated watermelon seedlings. DNA and low molecular weight RNA (4S, 5S) was removed by salt washes from the total ethanol-precipitated RNA. As analyzed by polyacrylamide/agarose gel electrophoresis (11), the bulk of ribosomal RNA was separated from polyadenylated mRNA by chromatography on oligo (dT)-cellulose. The overall yield of polyadenylated RNA was 3.0 mg per 600 pairs of cotyledons.

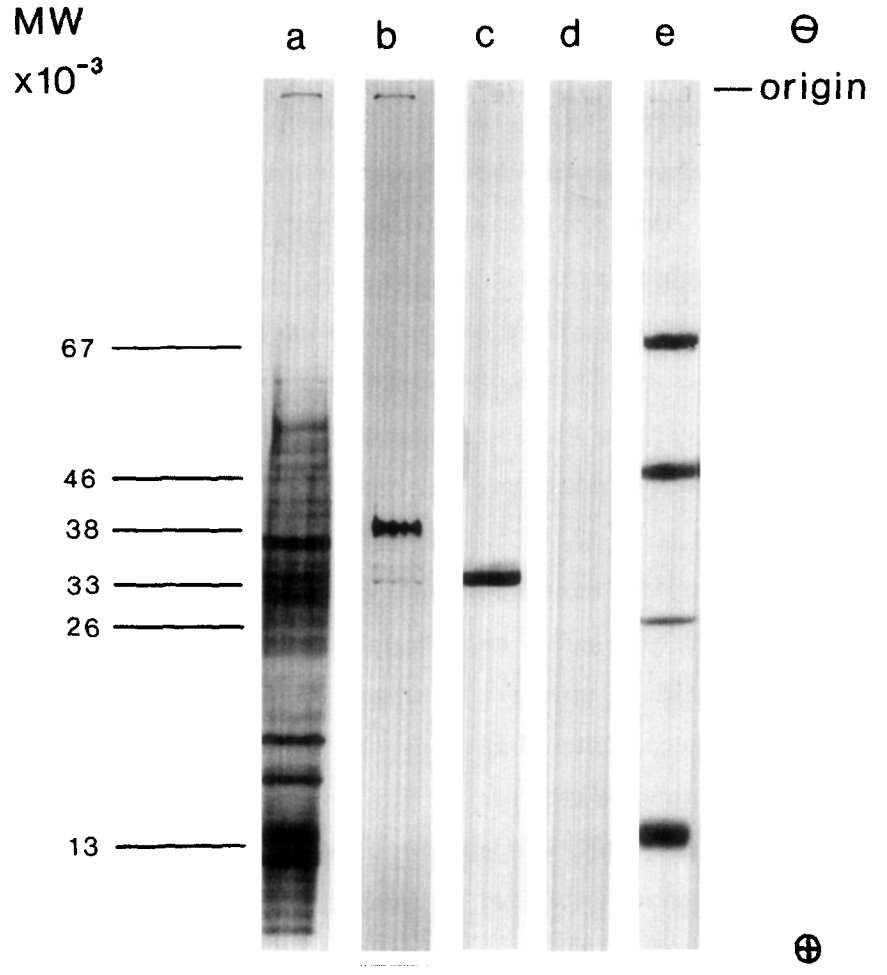

Fig.1: Characterization of polypeptides synthesized by watermelon polyadenylated mRNA. Analysis was by SDS-PAGE followed by protein stain (Coomassie Brilliant Blue R-250; c and e) or autoradiography (a,b and d). (a) [^{35}S]-Labelled polypeptides synthesized in the presence of watermelon mRNA. (b) Direct immunoprecipitation of (a) with antibodies against glyoxysomal MDH. (c) Purified authentic glyoxysomal MDH. (d) Molecular weight standards: cytochrome C (13 000), chymotrypsinogen (26 000), ovalbumin (46 000), and bovine serum albumin (67 000). MW = molecular weight.

Addition of this purified mRNA fraction to cell-free extracts derived from wheat germ in the presence of [^{35}S] methionine resulted in a 30-50-fold increase of incorporation of

radioactivity into hot trichloroacetic acid insoluble material (Tab.1) after 90 min of reaction. The rate of incorporation was proportional to the amount of mRNA added up to 6 µg per 50 µl of reaction mixture and proportional to incubation time up to 90 min. This stimulation of protein synthesis could be blocked by cycloheximide but not by chloramphenicol (Tab.1).

The products of cell-free translation were separated by SDS-PAGE and visualized by autoradiography. As shown in Fig. 1a, labelled peptides with molecular weights up to 100 000 were detected by SDS-PAGE, indicating the translation of rather long messengers.

Cell-free products were also analyzed immunochemically for the presence of antigenic determinants of glyoxysomal MDH. Upon termination of protein synthesis (90 min) and removal of polysomes by centrifugation, the supernatant was used for the immunoprecipitation of the newly synthesized radioactive glyoxysomal MDH. The precipitated material was analyzed by SDS-PAGE. Four labelled polypeptides were observed in autoradiographs (Fig. 1b). One of these comigrated with glyoxysomal MDH protein (Fig. 1c; subunit size: 33 000). The most intense band had a significantly higher molecular weight (38 000). No precipitation of labelled polypeptides was detected using preimmune antibodies from the same rabbit (Fig. 1d). Translation of polyadenylated mRNA from rat liver polysomes was also carried out with the wheat germ system; unlabelled glyoxysomal MDH was added and immunoprecipitated. Radioactive polypeptides were absent from precipitates analyzed by SDS-PAGE. Immunoprecipitation was also performed to test for any products with low endogeneous incorporation of label from the wheat germ extract in the absence of added mRNA

(Tab.1). No polypeptides could be detected by autoradiography. These control experiments demonstrated specific immunoprecipitation of labelled glyoxysomal MDH only in the presence of added messenger isolated from watermelon cotyledons.

DISCUSSION

The identification of different forms of glyoxysomal MDH is based upon the specifity of the antibody used. This antibody fraction was shown to interact specifically with the purified glyoxysomal, but not with the purified mitochondrial or a mixture of partially purified cytoplasmic MDH from watermelon cotyledons. In double diffusion experiments, the same antibody fraction precipitated a single protein in crude cotyledon extracts (2). This precipitate exhibited MDH activity and was serologically identical with the purified glyoxysomal isoenzyme (2). Thus, the antibody fraction used in this study was monospecific for glyoxysomal MDH.

Unspecific precipitation of labelled polypeptides was excluded by various controls including the use of preimmune gammaglobulins and precipitation of carrier isoenzyme from a wheat germ incubation mixture containing radioactive nascent rat liver polypeptides and unlabelled glyoxysomal MDH.

Incorporation of label into polypeptides, including polypeptides precipitated with antibodies against glyoxysomal MDH, was inhibited by cycloheximide but not by D(-)threochloramphenicol. Therefore, the glyoxysomal isoenzyme is synthesized *in vitro* by cytoplasmic 80 S ribosomes. The same result was indicated by *in vivo* experiments (2).

Since the cell-free synthesized polypeptide with a molecular weight of 38 000 precipitated with the antibody fraction, it obviously contains determinants of the glyoxysomal MDH.

This polypeptide is larger than subunits of the authentic isoenzyme and may represent a precursor protein with a signal peptide. This signal peptide may not be removed in the absence of watermelon cellular membranes. Larger precursor molecules of polypeptides destined for integration into cell organelles have been described by Dobberstein et al.(12). Detailed peptide analysis of the large polypeptide and its comparison to the peptide pattern of authentic glyoxysomal isoenzyme is in progress. The presence of the authentic isoenzyme in the cell-free supernatant indicated that the wheat germ extract must contain a component capable of removing the additional polypeptide chain from the larger precursor. This cleaving activity seems to be rather inefficient, since after 90 min of protein synthesis most of the label remains associated with the large molecular weight form. The difference in the molecular weights of precursor and authentic isoenzyme is larger than observed with precursor peptides investigated previously (13). This large size could mean that the removal of the additional entity from the glyoxysomal isoenzyme precursor is a process involving cleavage of more than a single peptide bond. The presence of two very faintly labelled bands between the precursor and the authentic form may represent different stages of such a process.

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