BIOSYNTHESIS OF GLYOXYSOMAL ENZYMES IN *NEUROSPORA CRASSA**

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INTRODUCTION

Little is known about the formation of glyoxysomes in comparison to the formation of other cell organelles such as mitochondria or chloroplasts. We do not have an answer for the basic question of how new glyoxysomes arise in the cell.

One possibility is that they originate *de novo* by a continuous process of differentiation from other cellular structures. A vectorial flow of membranes from the endoplasmic reticulum (ER) has been proposed.[1,2] A programmed segregation of membrane components would provide the information for the assembly of new glyoxysomes. According to this view new glyoxysomal proteins would be inserted into ER membranes by a cotranslational mechanism. An alternative view is that new glyoxysomes can only arise from preexisting ones, i.e. glyoxysomes would multiply by growth and division. Information for the assembly in this case would reside in the organelles themselves, as has been demonstrated for mitochondria and chloroplasts. In the latter case, glyoxysomes would have to be inherited; in the former case they could be formed completely anew. A major reason for our lack of information is the lack of suitable experimental systems. One possibly useful system for the study of glyoxysome biogenesis is Neurospora cells that are shifted from a medium containing sucrose to a medium containing acetate. The activities of key enzymes of the glyoxylate cycle are very low in cells grown on glucose or sucrose, but they increase by at least a factor of 30 within a few hours after the shift to acetate as carbon source.[3,4] Electron microscopy of cross-sectioned cells shows a parallel increase in structures with the characteristics of glyoxysomes.

We have started to investigate the biogenesis of Neurospora glyoxysomes by analyzing the synthesis and intracellular translocation of individual glyoxysomal matrix enzymes. These were compared to the biosynthesis and assembly of citrate synthase, a mitochondrial matrix enzyme which is also induced after the shift to acetate. The results suggest a post-translational transfer of glyoxysomal matrix enzymes into glyoxysomes. Hence, assembly of the glyoxysomal matrix

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may occur through specific insertion of newly synthesized completed polypeptide chains into preexisting glyoxysomes.

METHODS

Hyphae of *Neurospora crassa* (WT 74A) were grown in a solution containing Vogel's minimal medium[5] and 2% sucrose under vigorous aeration for 14 hr at 25°C. They were then harvested by filtration and resuspended in a solution containing Vogel's minimal medium and 40 mM sodium acetate. Aeration and temperature remained unaltered. Aliquots containing 100 ml of culture were withdrawn at various times after shifting the cells to the acetate-containing medium. Cells were harvested by filtration and ground with sand in a medium that contained 1 mM $MgCl_2$, 1% Triton X-100, and 10 mM Tris-HCl, pH 8.1. After grinding, the homogenate was centrifuged twice for 5 min at 5000 × g and once for 15 min at 17,000 × g, The resulting supernatant fraction was used to determine enzyme activities. For preparation of free and membrane-bound ribosomes, cycloheximide-treated cells were ground with a cell mill in a medium containing 0.88 M sucrose, 0.2 M NH_4Cl, 20 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5, and 0.05% heparin. Free and membrane-bound ribosomes were separated on discontinuous sucrose gradients and RNA was isolated from the different fractions.[6-9]

Malate synthase was isolated essentially following the procedure described for yeast[10]; citrate synthase and isocitrate lyase were isolated using published procedures.[11,12] The following enzyme activities were determined in cell extracts employing established methods: Isocitrate lyase,[12] malate synthase,[10] citrate synthase,[13] malate dehydrogenase,[14] and fumarase.[15] Antibodies against enzymes were raised in rabbits as described.[16]

Protein synthesis in cell-free systems and immunoprecipitation were performed as outlined elsewhere.[17,18] For labeling of proteins *in vivo*, cells were grown at a reduced concentration of sulfate (0.08 mM) and in the presence of 0.5 mCi/ℓ [^{35}S]sulfate (spec. radioactivity 10-1000 mCi/mol).

For electron microscopy, hyphae were converted into spheroplasts by incubating them with snail gut enzyme in sorbitol-containing medium.[17] Spheroplasts were collected by centrifugation and fixed for 12 hr in 0.1 M cacodylate buffer pH 7.4, 0.2 M sucrose, and 2.5% glutaraldehyde at 4°C. Pellets were rinsed three times with 0.2 M sucrose, 0.1 M cacodylate buffer, pH 7.4. Pellets were resuspended in 1% OsO_4, 0.1 M cacodylate, pH 7.4, 0.2 M sucrose and kept at 4°C for 1 hr. Cells were repelleted and treated with 1% uranyl acetate, and 0.2 M sucrose for 1 hr. Samples were then dehydrated in ethanol and embedded in Epon 812. Thin sections were cut in a LKB ultrotome III and stained with magnesium uranyl acetate and lead. They were viewed in a Siemens Elmiscop 102.

RESULTS

When a Neurospora culture was shifted to an acetate-containing medium after growing for 16 hr on sucrose, cell growth stopped for several hours. The cells then entered a new growth period with the maximum growth rate at about 14–16 hr after the shift. After a further 8 hr the cells stopped growing, probably because the acetate source was exhausted (FIG. 1).

In the electron microscope, the appearance of particles with the characteris-

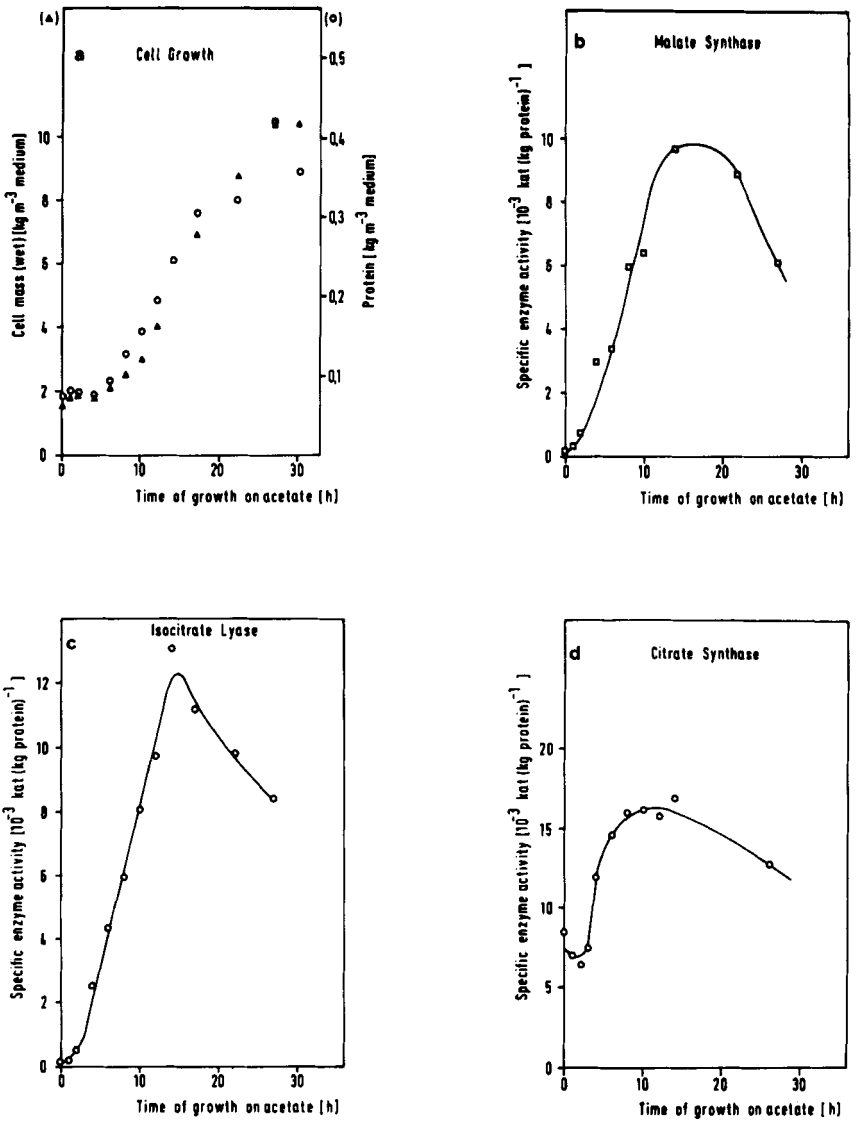
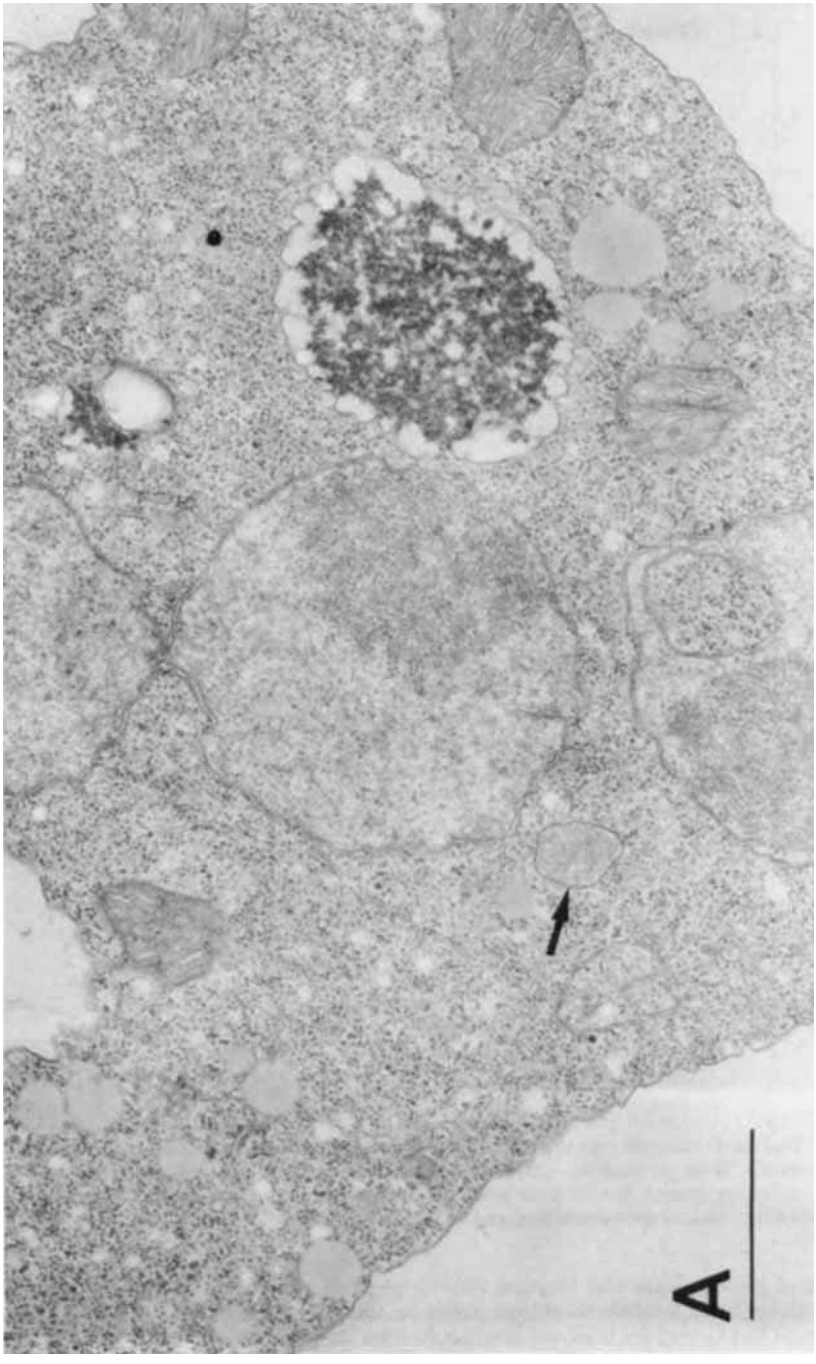


FIGURE 1. Induction of various enzyme activities by a shift of Neurospora cells from a medium containing sucrose to a medium containing acetate as a carbon source. Cells were grown for 14 hr on sucrose, harvested, and resuspended in acetate-containing medium. After further growth for the time periods indicated, aliquots were withdrawn. Wet mass and total protein were determined and enzyme activities were measured in cell extracts.

tics of glyoxysomes can be seen after 7-24 hr of growth on acetate (FIG. 2). The particles have a single membrane and an unstructured dense matrix. However, organelles bound by a single membrane can also be observed in cells grown on sucrose, although at much lower frequency.

FIGURE 1, b & c, shows that the levels of the key enzymes of the glyoxylate cycle,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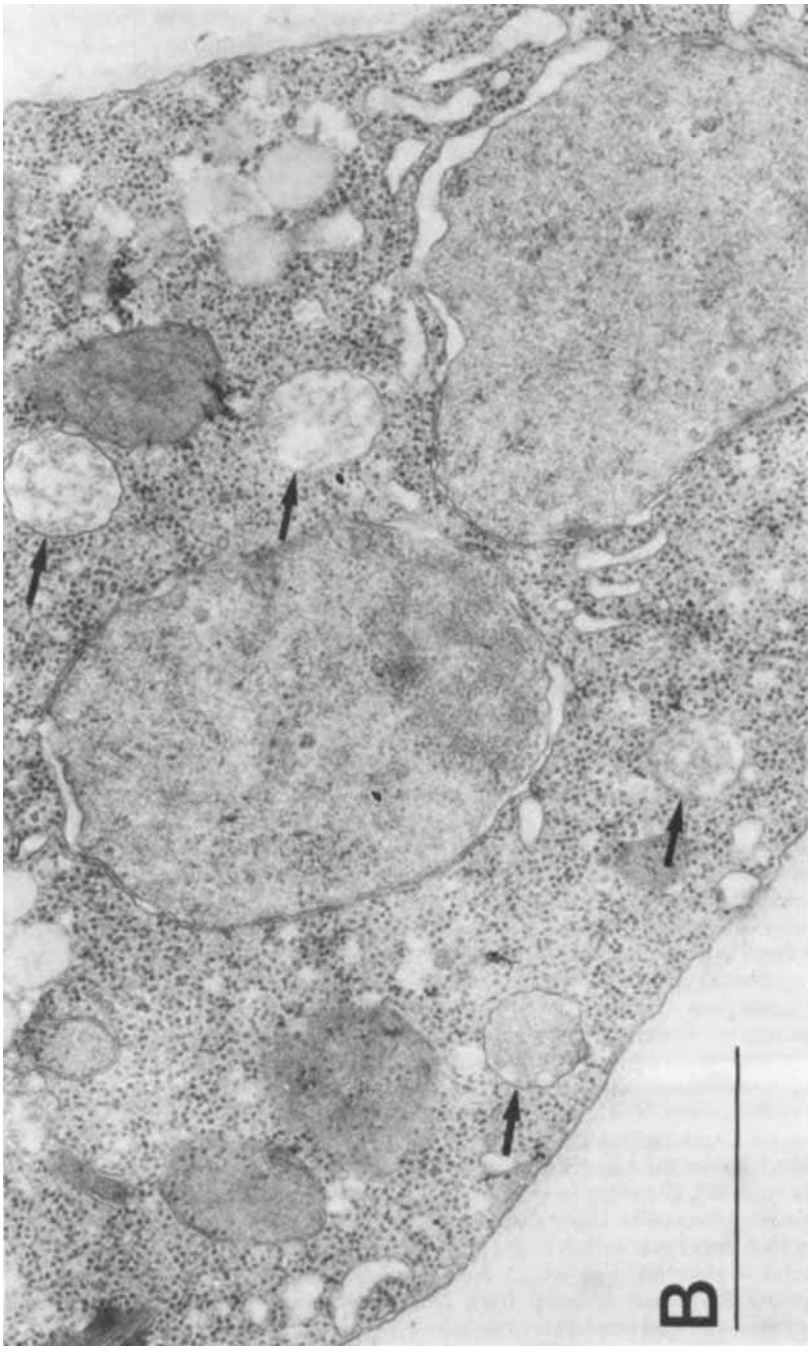

FIGURE 2. Thin sections of spheroplasts prepared from Neurospora cells grown on sucrose or acetate. **A**, cells grown on sucrose for 14 hr; **B**, cells grown on sucrose for 14 hr and then on acetate for another 14 hr. Arrows point to microbody structures.

isocitrate lyase and malate synthase, were practically zero in the cells grown on sucrose. After shift of the cells to acetate, there was a lag period of about 1 hr, followed by a rapid increase of the two enzyme activities. They reached a maximum after about 14 hr on acetate. From the specific activities of these enzymes in whole cell extracts and in the purified state the enzymes can be calculated to make up 1-2% of the total cellular protein after maximal induction.

FIGURE 1 also shows the behavior of citrate synthase. This enzyme is also involved in the formation of carbohydrates from acetate, but in fungi it is not localized in glyoxysomes, but in mitochondria.[19,20] Citrate synthase is present in sucrose grown cells at a high level, and is induced only about twofold after a lag period of about 1 hr, more rapidly than malate synthase and isocitrate lyase.

Malate dehydrogenase and fumarase, like citrate synthase, are clearly present in sucrose-grown cells and, after a lag of about 1 hr, are induced in their specific activity by factors of about 2 and 15, respectively. Both enzymes have been shown not to be present in glyoxysomes of Aspergillus or Neurospora.[19,20]

Citrate synthase present in cells grown on acetate was found to be identical (or very similar) to the enzyme present in mitochondria from uninduced cells. An antibody obtained against mitochondrial citrate synthase from cells grown on sucrose was employed to determine whether the enzyme from induced cells was antigenically related. The inhibition of citrate synthase activity by this antibody in extracts from sucrose and acetate grown cells was identical. Furthermore, citrate synthase from both induced and uninduced cells was inhibited by ATP to the same extent; 10 mM ATP gave an inhibition of about 70%. Finally, citrate synthase immunoprecipitated from induced cells and from uninduced cells yielded a band with the same apparent molecular weight of 45,000 on SDS-containing gels. These results suggested that the induced citrate synthase is the same as that presented in uninduced cells, i.e. an induction of the mitochondrial enzyme occurs.

Growth on acetate therefore does not only lead to the formation of glyoxysomes but also to a distinct alteration of mitochondrial function. The observed lag period of about 1 hr represents the time needed by the cell to begin to reorganize its enzymic outfit and to form glyoxysomes. Electron micrographs show that glyoxysomes are less abundant 7 hr after shifting to acetate than after 14 hr or 25 hr. Growth of cells (cell mass and total cellular protein) again lags behind the appearance of glyoxysomal enzymes.

In order to establish that the enzymes are not induced by enzyme activation but that there is a real *de novo* synthesis during growth on acetate, the levels of malate synthase, isocitrate lyase, and citrate synthase were determined with specific antibodies.

Immunoprecipitates were obtained from extracts of cells grown either on sucrose or sucrose followed by acetate. The culture media contained [^{35}S]sulfate so that cysteine and methionine residues in proteins were labeled with a defined specific radioactivity. The immunoprecipitates were analyzed by SDS gel electrophoresis and autoradiography.

FIGURE 3 shows the bands corresponding to malate synthase (M_r 59,000) and isocitrate lyase (M_r 67,000) to be present in the case of acetate-grown cells but not with sucrose-grown cells. Citrate synthase (M_r 45,000) is present in both types of cells, but in a larger proportion in the acetate-grown cells.

In order to determine in which form these proteins are synthesized, poly-A-containing RNA was isolated from Neurospora cells and translated in a reticulocyte lysate. The precursor proteins formed were immunoprecipitated and analyzed by SDS gel electrophoresis and autoradiography. The precursor

proteins of malate synthase and isocitrate lyase have the same apparent molecular weight as their mature counterparts (FIG. 4). In the case of isocitrate lyase, synthesis in the reticulocyte lysate was also carried out with *N*-formyl-[^{35}S]methionyl-tRNA.[16] A labeled product was obtained which had the same apparent molecular weight as the mature protein. Apparently, the original translation product was not proteolytically processed at the amino terminus. The intracellular transfer of these enzymes therefore does not involve the removal of a "signal" type sequence.

In contrast to these two glyoxysomal enzymes, citrate synthase is synthesized as a larger precursor with an additional sequence of about 2000 daltons (FIG. 4).

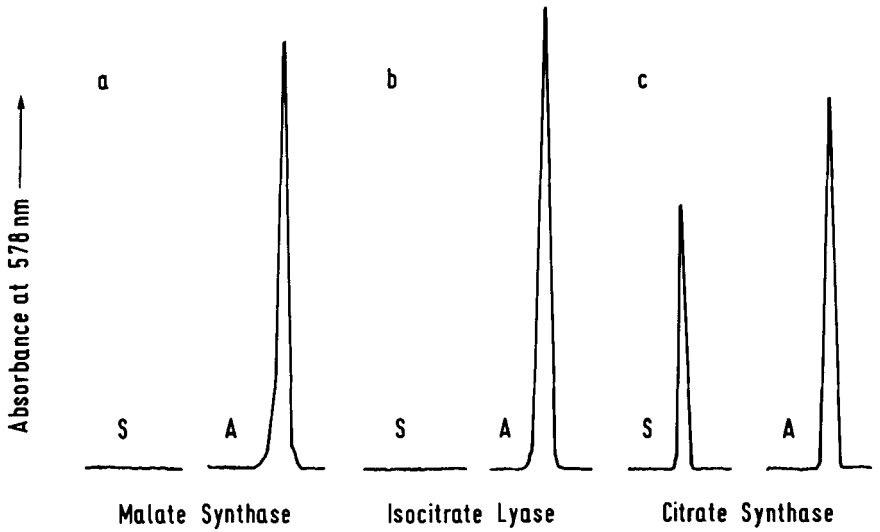

FIGURE 3. Immunoprecipitation of malate synthase, isocitrate lyase, and citrate synthase from Neurospora cells grown on sucrose or on acetate. Neurospora cells were grown for 14 hr on sucrose or for 14 hr on sucrose and then for 14 hr on acetate, in medium containing [^{35}S]sulfate. After cells were harvested, a total cell extract was prepared and immunoprecipitation with antibodies against the three enzymes was performed from aliquots. Immunoprecipitates were subjected to electrophoresis in the presence of SDS, and the dried gels were autoradiographed. The bands corresponding to the individual enzymes were quantitated by densitometry at 578 nm.

Citrate synthase is imported into mitochondria in a post-translational process involving the cleavage of the additional sequence.[21,22]

Are glyoxysomal enzymes synthesized by free or membrane-bound ribosomes? Two different approaches were used to answer this question. In the first one, free and membrane-bound ribosomes were prepared by equilibrium density gradient centrifugation. Read-out synthesis was carried out with these ribosomes after resuspending them in a 100,000 × g supernatant of a reticulocyte lysate. Isocitrate lyase was found to be synthesized only by free ribosomes (FIG. 5). In the second approach, free and membrane-bound ribosomes were separated and the RNA was isolated. These RNAs were translated in reticulocyte lysate and malate synthase was immunoprecipitated. Only the RNA from free ribosomes was able to promote synthesis of malate synthase. These findings suggest that the glyoxyso-

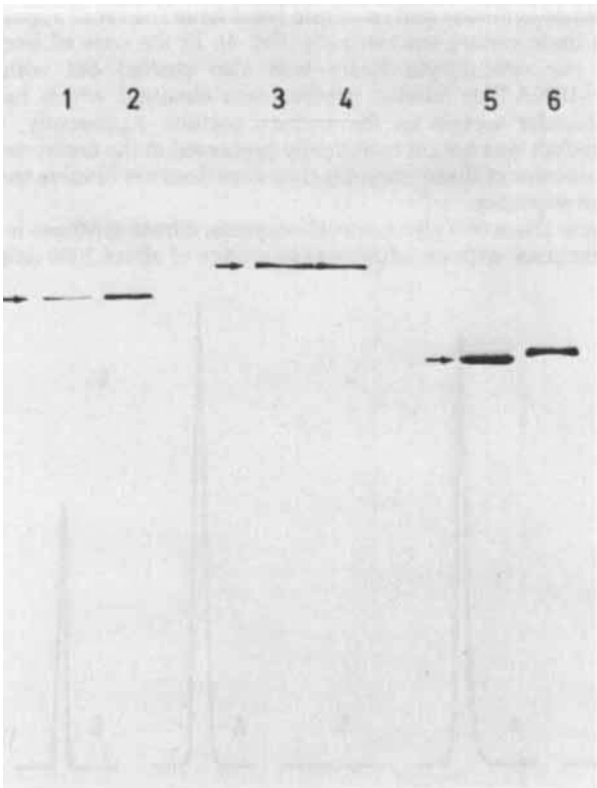

FIGURE 4. Synthesis of malate synthase, isocitrate lyase, and citrate synthase in cell-free systems. RNA was extracted from cells grown on acetate and translated in reticulocyte lysates in the presence of [^{35}S]methionine. Malate synthase and isocitrate lyase were immunoprecipitated. These immunoprecipitates were analyzed on SDS-containing gels parallel to immunoprecipitates obtained from extracts of Neurospora cells grown on acetate and [^{35}S]sulfate. Citrate synthase was synthesized in a homologous cell-free system from Neurospora and immunoprecipitated. This immunoprecipitate was also run beside an immunoprecipitate from cells grown on [^{35}S]sulfate. Citrate synthase was also synthesized in reticulocyte lysates programmed with RNA from cells grown on sucrose or acetate and again a larger precursor was found (not shown). The precursor in the reticulocyte lysate, however, was partly sensitive to endogenous proteases and aside from the band with M_r 47,000, bands with lower M_r were consistently observed. *Lanes 1,2*: malate synthase (M_r 59,000); *Lanes 3,4*: isocitrate lyase (M_r 67,000); *Lanes 5,6*: citrate synthase (mature form M_r 45,000, precursor M_r 47,000); *Lanes 1,3,5*: mature enzymes; *Lanes 2,4,6*: precursor proteins, synthesized in cell-free systems Arrows point to the position of the band of stained mature enzyme on the gel.

mal enzymes are made on free ribosomes and are released into the cytosolic compartment.

Intracellular transfer of glyoxysomal enzymes was studied in a system consisting of the supernatant fractions of reticulocyte lysates, which contained newly synthesized enzyme precursors, and a particle fraction containing glyoxysomes. After incubating lysates and particles together, the particulate fraction was reisolated. It was treated with proteinase K to digest any protein adsorbed

but not transferred across membranes. Then isocitrate lyase was immunoprecipitated. FIGURE 6 shows that under these conditions isocitrate lyase becomes associated with the particulate fraction. In a control experiment, the same protocol was followed using a particle fraction from cells grown on sucrose, where very few glyoxysomes are present. In this case, isocitrate lyase did not become associated with the particle fraction.

These findings suggest that glyoxysomal enzymes are transferred into glyoxysomes in a post-translational manner. They appear to be synthesized on free ribosomes, released into the cytosol, and then taken up into glyoxysomes without a proteolytic processing step being involved.

DISCUSSION

The experiments described here are consistent with the view that glyoxysomes multiply by a self-assembly mechanism, triggered by the specific insertion

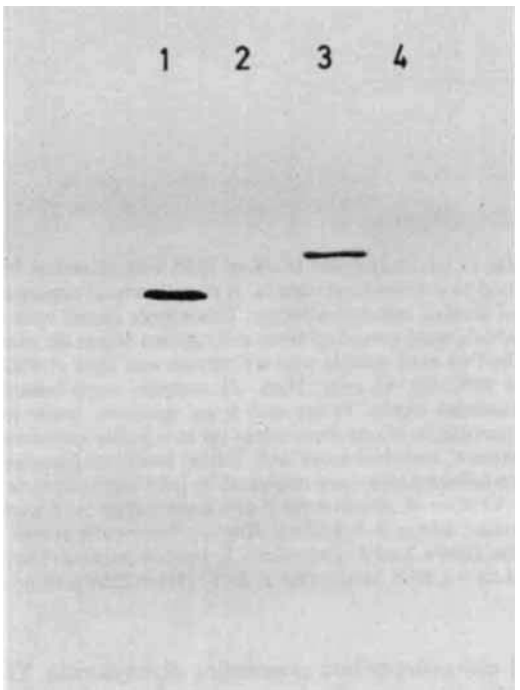

FIGURE 5. Synthesis of malate synthase and isocitrate lyase by free ribosomes. Free and membrane-bound ribosomes were separated by density gradient centifugation. The ribosomes were employed in a cell-free read out system or the RNAs were extracted from both types of ribosomes. The extracted RNAs were translated in reticulocyte lysates in the presence of [^{35}S]methionine, and malate synthase was immunoprecipitated from both lysates. In the read out experiments, free and bound ribosomes were resuspended in postribosomal supernatants from reticulocyte lysates, and the mixtures were incubated in the presence of [^{35}S]methionine. Isocitrate lyase was immunoprecipitated from these samples. Immunoprecipitates were subjected to gel electrophoresis in the presence of SDS and the dried gels were autoradiographed. *Lanes 1,2*: malate synthase; *Lanes 3,4*: isocitrate lyase; *Lanes 1,3*: free ribosomes; *Lanes 2,4*: membrane-bound ribosomes.

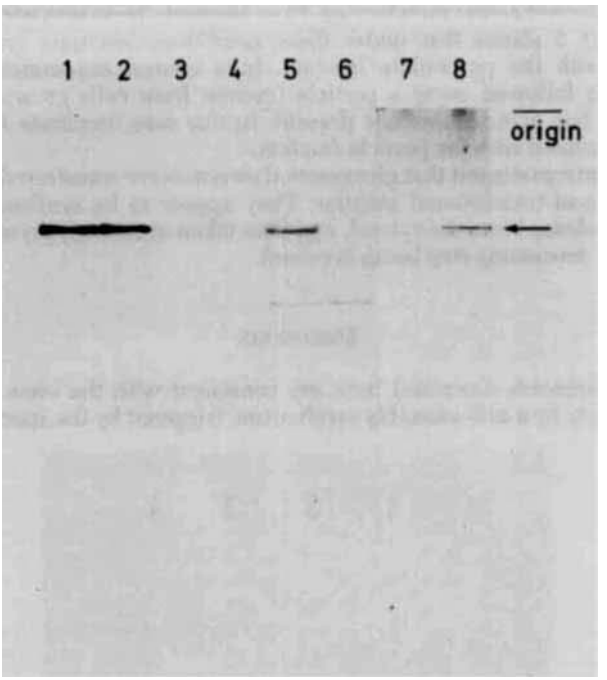

FIGURE 6. Transfer of isocitrate lyase *in vitro*. RNA was extracted from cells grown on acetate and translated in a reticulocyte lysate. A postribosomal supernatant was prepared from this lysate and divided into two aliquots. These were mixed with 12 min, 17,000 × g particle fractions which were prepared from cells grown either on sucrose or on acetate. After mixing, one half of each sample was withdrawn and kept at 0°C. The other halves were incubated at 25°C for 60 min. Then all samples were centrifuged to separate supernatant and particles again. Triton and 5 μg isocitrate lyase were added to the supernatants. The particle fractions were taken up in a buffer containing 0.44 M sucrose, treated with proteinase K, and then lysed with Triton. Isocitrate lyase was immunoprecipitated and the immunoprecipitates were analyzed by gel electrophoresis and autoradiography. *Lanes 1-4*: incubation of reticulocyte lysate supernatant with particle fraction from cells grown on sucrose; *Lanes 5-8*: particle fraction from cells grown on acetate; *Lanes 1,2,5,6*: supernatants; *Lanes 3,4,7,8*: proteinase K treated particle fraction; *Lanes 1,3,5,7*: incubation at 0°C; *Lanes 2,4,6,8*: incubation at 25°C. (From Zimmerman & Neupert.[18] Used with permission.)

of newly formed components into preexisting glyoxysomes. Though very rare, glyoxysome-like structures can also be seen in cells in which the enzymes of the glyoxylate pathway are not present or are only present in minimal amounts. There could be low constitutive expression of glyoxysomes or related structures, sufficient to allow propagation of organelles during cell division. These few organelles could then store the information for the rapid formation of a high number of glyoxysomes when cells have to rely on acetate as carbon source. However, though consistent with such a self-assembly model, these data do not exclude a *de novo* formation of glyoxysomes. From electron microscopic observations it was suggested that microbodies could arise by budding from the endoplasmic reticulum.[1,2] Biochemical evidence for such a mechanism is scarce.[23] Contradictory findings on the involvement of rough endoplasmic reticulum in

the synthesis of glyoxysomal enzymes have been published.[24,25] Recent data including those presented here indicate that enzymes of the glyoxysomal matrix[16,26] as well as the peroxisomal matrix[27,28] are made on free ribosomes and are post-translationally transferred via the cytosolic compartment across the membrane of the microbody. In this context it is important to note that glyoxysomal and peroxisomal matrix enzymes are synthesized with the same length as they show in their mature, enzymatically active form. This has been demonstrated for catalase and uricase,[27,28] for isocitrate lyase,[16] and for malate synthase.[26,29] Apparently, a "signal" type sequence is not removed during transmembrane transfer of the precursor protein. This is in contrast to the cotranslational transfer of secreted proteins across the membrane of the endoplasmic reticulum.[30,31] In the latter case, with very few exceptions, precursor proteins synthesized in cell free systems have amino-terminal extensions of about 20–30 amino acids. However, a larger precursor has also been reported for a glyoxysomal protein, namely malate dehydrogenase.[29,32] It should be pointed out that determination of amino-terminal sequences of the precursor and mature forms of glyoxysomal enzymes must be carried out before final conclusions on processing events can be made. Also, the possible glycoprotein nature of some of these enzymes[24,29] might cause some complications. On the other hand, malate synthase and isocitrate lyase from *Ricinus* were recently reported not to be glycoproteins.[33]

Support for the view that precursors of glyoxysomal matrix enzymes are transferred into preexisting organelles post-translationally comes from the observation that large pools of glyoxysomal enzymes accumulate in the cytosolic fraction of germinating seeds at certain stages of development. These extraglyoxysomal precursors are later transferred into the glyoxysomes.[34–36]

Although all these observations support a self-assembly mechanism of microbodies, it cannot be excluded that the membranes of the microbodies arise by membrane flow from other cell organelles, especially from the ER. Actually, such a mechanism has been proposed for rat liver peroxisomes[28] to account for electron microscopy data which suggest some continuity between ER and glyoxysomes. Further studies on the assembly of glyoxysomes will have to rely on various *in vitro* systems to analyze individual steps in this process, but these are severely hampered by difficulties in isolating intact glyoxysomes in reasonable yield and sufficient purity.

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DISCUSSION OF THE PAPER

R. R. THEIMER (*University of Munich, FRG*): I do not see any discrepancy between what you think and what we think. Your last slide showed that there is, in the sucrose-grown cells of the wild type, apparently no incorporation of the isocitrate lyase into the particles. Your precursor particles are then obviously not present, because the isocitrate lyase is not incorporated.

W. NEUPERT (*University of Göttingen, FRG*): No, the experiments only say that they are present to such a low degree that a crude experiment could not detect them. You have seen that the uptake system is not very efficient.

THEIMER: But what difference does it make that these precursor particles derive from the ER and subsequently take up protein synthesized in the cytoplasm? Then, they may differentiate or mature to glyoxysomes. So, ultimately they are derived from the ER. Catalase was found in vesicles that were in the sediment together with the ER membranes. If you induce the microbody enzymes, these vesicles appear to mature and to increase in density. It was also shown that there are small vesicles already showing DAB-staining when the leaves are in the first stages of greening. Why do you oppose this hypothesis that

small vesicles are budding off the ER and then finally maturing by taking up these enzymes from the cytoplasm. I do not see a discrepancy.

NEUPERT: I think we are confusing the issue now. Would you call everything endoplasmic reticulum which is a vesicle from somewhere in the cell?

THEIMER: Why could they not derive from the endoplasmic reticulum?

NEUPERT: What I am offering is a very simple explanation that would be sufficient to explain the observed facts. If there is a continuity of peroxisomes within cells and if there is continuity of organelles which have the identity or the potential identity of glyoxysomes, I do not see any reason to postulate this ER, which complicates the whole story.

THEIMER: I do not see any complication; your suggestion is more complicated than the other because you need the phospholipid exchange protein for the flux of lipids; and how do the proteins find the right organelle? Furthermore, we know that there is a sidedness in the case of membrane phospholipids.

NEUPERT: We should consider only the proteins, because the transport of these lipids is very complicated and virtually unknown. If you have this ER-derived mechanism, you have to have at least two steps of recognition: a signal for the membrane-bound ribosomes, and an additional step of recognition which serves to segregate the newly made membrane proteins into glyoxysomes and other organelles.

THEIMER: But then you would have to produce the recognition proteins in the cytoplasm and to incorporate this recognition proteins into the right organelles.

NEUPERT: Yes.

THEIMER: Why don't you incorporate the microbody recognition proteins into the mitochondria by accident?

NEUPERT: I mean the simple thing is the continuity of membranes. It is obvious that we have the information for assembly of the mitochondria in the mitochondrial membrane, and this information is passed on from one cell to the other by passing on the mitochondria. This does not have anything to do with DNA, it is just the information in the membrane. What I am proposing is that exactly the same is the case with the glyoxysomes.

E. GONZALEZ (*University of California, Los Angeles*): Did you show that this translocation step for isocitrate lyase into glyoxysomes is an energy-requiring step?

NEUPERT: I do not know.

GONZALEZ: Do you have any other evidence for translocation of glyoxysome-specific proteins into the mature glyoxysome?

NEUPERT: No.

A. B. NOVIKOFF (*Albert Einstein College of Medicine, Bronx, NY*): Do you feel that tunicamycin is all that specific an inhibitor of synthesis of all glycoproteins?

NEUPERT: It has been shown for Neurospora and it works also in yeast. I am not aware of an exception. Also, we should not mix *O*-glycosylation and *N*-glycosylation.

H. BEEVERS (*University of California, Santa Cruz*): Your import experiment was done with a crude fraction; can you be sure that the isocitrate lyase went into the glyoxysomes?

NEUPERT: No. It was very difficult because of the fragility of the organelles. But we have a control with organelles from the sucrose-grown cells, where we had all the other organelles but practically no glyoxysomes.

BEEVERS: But you did not try an incorporation into purified mitochondria from acetate-grown cells, just as a sort of negative control?

NEUPERT: It would again mean that you have to purify the mitochondria and make sure that you do not have any glyoxysomes there.