Eur. J. Biochem. *112*, 225–233 (1980)

Biogenesis of Glyoxysomes

Synthesis and Intracellular Transfer of Isocitrate Lyase

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(Received August 26, 1980)

Biosynthesis of isocitrate lyase, a tetrameric enzyme of the glyoxysomal matrix, was studied in *Neurospora crassa*, in which the formation of glyoxysomes was induced by a substitution of sucrose medium by acetate medium.

1. Translation of *Neurospora* mRNA in reticulocyte lysates yields a product which has the same apparent molecular weight as the subunit of the functional enzyme. Using *N*-formyl[^{35}S]methionyl-$tRNA_f^{Met}$ as a label, the translation product shows the same apparent size which indicates that the amino terminus has no additional 'signal'-type sequence.

2. Read-out systems employing free and membrane-bound polysomes show that only free ribosomes are active in the synthesis of isocitrate lyase.

3. Isocitrate lyase synthesized in reticulocyte lysate is released into the supernatant and is soluble in a monomeric form. It interacts with Triton X-100 to form mixed micells in contrast to the functional tetrameric form.

4. Transfer of isocitrate lyase synthesized *in vitro* into isolated glyoxysomes is suggested by results of experiments in which supernatants from reticulocyte lysates are incubated with a particle fraction isolated from acetate-grown cells. No transfer occurs when particles from non-induced cells are employed. Resistance to added proteinase is used as a criterion for transmembrane transfer.

The data support a post-translational transfer mechanism for isocitrate lyase. They suggest that isocitrate lyase passes through a cytosolic precursor pool as a monomer and is transferred into glyoxysomes.

The reactions involved in the formation of glyoxysomes are largely unknown. There are two hypotheses on the mechanism of glyoxysome formation. According to one hypothesis, they originate by budding of the endoplasmic reticulum. The proteins of the glyoxysomal matrix are thought to be synthesized on membrane-bound ribosomes of the endoplasmic reticulum, cotranslationally translocated and eventually channeled into the nascent glyoxysomes [1–4]. The second hypothesis implies a post-translational mechanism for protein insertion, i.e. the proteins enter pre-existing glyoxysomes. Glyoxysomes thus would be formed by growth and division. A variation of the second hypothesis includes the view that glyoxysomes bud from the endoplasmic reticulum but that the matrix components are added to the completed organelles. Work has been presented which tends to support the first hypothesis [1–4]. On the other hand, it seems possible that glyoxysomes are formed in a similar way as other cell organelles such as mitochondria, chloroplasts and peroxisomes. Recent work has shown that newly synthesized proteins can enter these organelles in a post-translational step, i.e. synthesis and intracellular translocation are separate events [5–16]. It was documented that precursors of organelle proteins exist outside the organelles. Whenever investigated, the precursors were detected in the cytosol fraction. In several cases it was shown that these precursors are transferred into the organelles, both in studies *in vivo* and *in vitro* [6–10, 12].

Neurospora cells grown in the presence of sucrose have a very low capacity to utilize acetate as a carbon and energy source. However, after a shift to an acetate-containing medium, they start to form glyoxysomes and the enzymes of the glyoxylate cycle which enables them to grow well on acetate [17–23]. The sucrose-to-acetate shift of *Neurospora* therefore is a suitable experimental system to study glyoxysome biogenesis, comparable to that of germinating seeds of fat-storing plant species [24–26].

Here we report on synthesis and translocation of a glyoxysomal matrix enzyme, isocitrate lyase. It is one of the enzymes of the glyoxylate cycle. The enzyme from various sources has been described to be a tetramer, consisting of four identical subunits with a molecular weight of $64-67 \times 10^3$ [27–29]. We report here

Enzyme. Isocitrate lyase (EC 4.1.3.1).

that the enzyme is synthesized by free polysomes. The primary translation product has the same size as the functional subunit as judged by electrophoresis in gels containing dodecylsulfate. The extra-glyoxysomal form is a monomer which interacts with detergent. Isocitrate lyase synthesized in a heterologous cell-free system is transferred *in vitro* into a particle fraction containing glyoxysomes in such a way that part of the newly formed protein becomes resistant to proteinase. The data are interpreted to suggest that at least this glyoxysomal matrix enzyme reaches its functional location in a post-translational transfer process. The precursor form appears to migrate through the cytosolic compartment.

MATERIALS AND METHODS

Cultivation of Neurospora *Cells and Cell Fractionation*

Neurospora crassa (wild type 74A) was grown under the following conditions.

a) A solution containing Vogel's minimal medium [30] and 2% sucrose was inoculated with 10^9 conidia/l and hyphae were grown under vigorous aeration at 25 °C for 16 h.

b) Hyphae were grown as in a) for 12 h, then harvested by filtration. The hyphae were resuspended in Vogel's minimal medium containing 40 mM sodium acetate, grown for further 4 h and then harvested.

Cells were fractionated either by grinding with sand [31] or by homogenisation of spheroplasts [8] in sucrose medium containing 0.44 M sucrose, 1 mM EDTA, 30 mM Tris/HCl, pH 7.5. To obtain a particle fraction containing glyoxysomes, the homogenate was first centrifuged twice for 5 min at $5000 \times g$ and then the supernatant for 12 min at $17000 \times g$. The pellet was resuspended in sucrose medium and once more centrifuged under the same conditions. Membrane-bound and free polysomes were prepared as reported elsewhere [32].

Isolation of Isocitrate Lyase

Isocitrate lyase was isolated from cells grown on acetate following the procedure of Johanson et al. [33]. Antibodies against the purified protein were raised in rabbits by injecting four times 0.5 mg in complete Freund's adjuvant at weekly intervals. One week after the last injection, blood was drawn from an ear vein and the immunoglobulin fraction was prepared by ammonium sulfate precipitation. Limited proteolysis of the immunoprecipitates was carried out after solubilizing immunoprecipitates in 2% sodium dodecylsulfate, 10 mM Tris/HCl, pH 7.5 as described previously [8].

Transfer Experiments in vitro

The $17000 \times g$ particle fractions obtained from spheroplasts of cells grown on sucrose or acetate were resuspended in the postribosomal supernatant from a reticulocyte lysate [8], which was supplemented with 0.44 M sucrose and 0.1 mg/ml cycloheximide. The reticulocyte lysate had been incubated with [^{35}S]-methionine, poly(A)-containing RNA from *Neurospora* cells grown on acetate and the necessary co-factors to support protein synthesis [32, 34, 35]. Transfer experiments were carried out for 60 min at 25 °C. Then the particle fraction was reisolated by centrifugation for 12 min at $17000 \times g$. After solubilisation with sucrose medium containing 1% Triton X-100 and 0.3 M KCl, direct immunoprecipitation was carried out using isolated isocitrate lyase (10 µg/ml) as a carrier for 12 h at 4 °C.

Gel Electrophoretic Analysis

Polyacrylamide gel electrophoresis in the presence of dodecylsulfate was carried out on vertical gels according to Laemmli [36]. Acrylamide/bisacrylamide concentrations of 10%/0.12% (w/v) were employed. Horizontal slab gel electrophoresis was performed as described [7]. Other methods have been described in earlier publications [5, 7, 8, 32].

RESULTS

Identification of Isocitrate Lyase by Immunoprecipitation

Isocitrate lyase isolated from cells grown in the presence of acetate displays on gels containing dodecylsulfate an apparent molecular weight of 65000. A minor band with a slightly lower molecular weight is also present (Fig. 1, lane 2). Antibodies against this enzyme precipitate a single protein band from whole cell extracts of *Neurospora* with an apparent molecular weight of 67000. This band is prominent on the stained gel (lane 1). Besides this, heavy and light chains of immunoglobulins are stained. To further demonstrate the specificity of the antibody, immunoprecipitation was carried out with an extract from cells grown in the presence of [^{35}S]sulfate. The autoradiograph shows a single band of M_r 67000 (lane 3). The protein can only be precipitated from cells grown on acetate but not from cells grown on sucrose (lane 4).

It is concluded from these findings that the subunit molecular weight of isocitrate lyase is 67000 and that during enzyme preparation the protein is altered in such a way that the apparent molecular weight is somewhat reduced, possibly by proteolytic degradation. A very similar behaviour has been reported for rat liver peroxisomal catalase, the subunit of which has an apparent molecular weight about

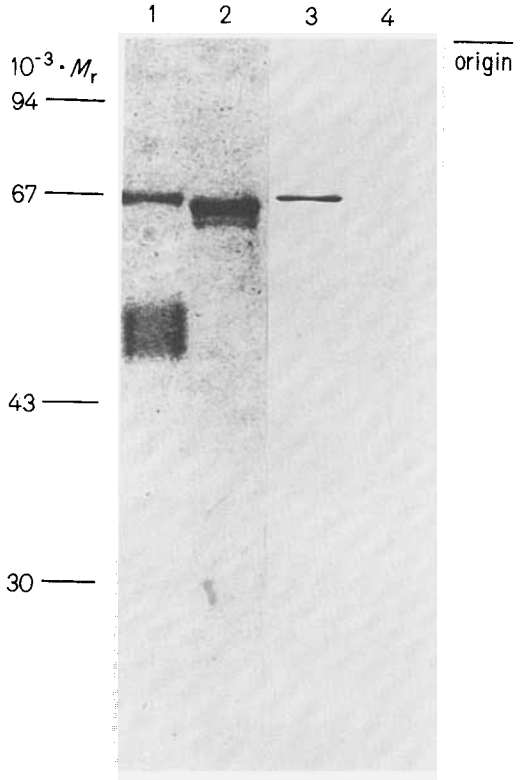

Fig. 1. *Immunoprecipitation of isocitrate lyase and analysis by polyacrylamide gel electrophoresis in the presence of dodecylsulfate.* Lane 1: isocitrate lyase was immunoprecipitated from an extract of *Neurospora* cells grown on acetate; Coomassie blue staining. Lane 2: purified isocitrate lyase from acetate grown cells; Coomassie blue staining. Lane 3: isocitrate lyase was immunoprecipitated from an extract of cells grown on acetate in the presence of [^{35}S]sulfate; autoradiograph. Lane 4: isocitrate lyase immunoprecipitated from an extract of cells grown on sucrose in the presence of [^{35}S]sulfate; autoradiograph. The molecular weight scale was obtained by coelectrophoresis of marker proteins

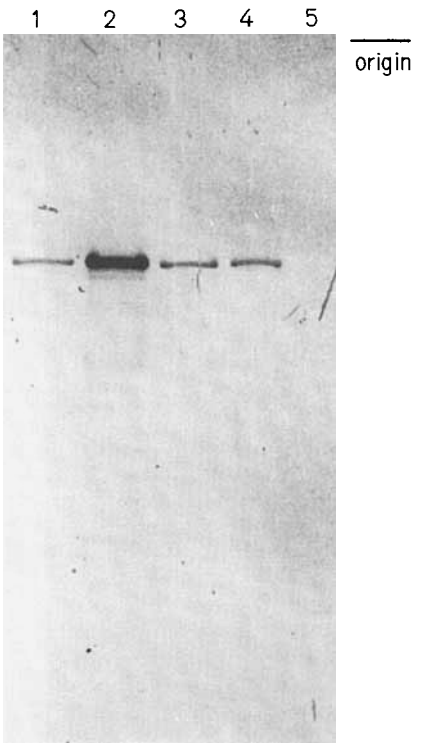

Fig. 2. *Synthesis of isocitrate lyase in reticulocyte lysates programmed with* Neurospora *poly(A)-containing RNA.* Poly(A)-containing RNA was isolated from cells grown on acetate or in sucrose. It was employed to program nuclease-treated reticulocyte lysates from rabbits [35]. Protein synthesis was carried out for 60 min. The supernatants of these lysates obtained after centrifugation for 1 h at 166000 × *g* were subjected to immunoprecipitation with antibodies to purified isocitrate lyase, using Sepharose-bound protein A. Immunoprecipitates were electrophoresed on polyacrylamide gels and the dried gels were autoradiographed. Lane 1, 4: reference; isocitrate lyase immunoprecipitated from cells grown on acetate in the presence of [^{35}S]sulfate. Lane 2: immunoprecipitate from reticulocyte lysate programmed with RNA from cells grown on acetate; labelling with [^{35}S]methionine. Lane 3: as in 2, but labelling with formyl[^{35}S]methionyl-tRNA. Lane 5: immunoprecipitate from reticulocyte lysate programmed with RNA from cells grown on sucrose; labelling with [^{35}S]methionine

2000 smaller as compared to the immunoprecipitated protein [15]. Isocitrate lyase is not present in measurable amounts in cells grown on sucrose but is induced to a relatively high level when cells are grown on acetate (about 2% of total cellular protein).

Isocitrate lyase from cucumber cotyledons was reported to contain carbohydrate [29]; other workers could not confirm this and conclude that isocitrate lyase is not a glycoprotein [37, 38]. Using the periodate/Schiff's stain [39] no carbohydrate was detected in the isolated or immunoprecipitated *Neurospora* enzyme (not shown here).

Synthesis of Isocitrate Lyase in Rabbit Reticulocyte Lysates

Poly(A)-containing RNA from *Neurospora* grown on sucrose and acetate, respectively, were employed to direct protein synthesis in rabbit reticulocyte lysates. Labelling of proteins was carried out with [^{35}S]-methionine. Isocitrate lyase was immunoprecipitated from postribosomal supernatants. Messenger RNA from cells grown on acetate directs the synthesis of a protein with the same apparent molecular weight as that of the authentic protein (Fig. 2, lanes 1 and 2). The messenger RNA from cells grown on sucrose does not promote synthesis of isocitrate lyase *in vitro* (lane 5). This latter finding excludes the possibility that an isoenzyme present in cells grown on sucrose [19, 20, 23, 40] interferes with the glyoxysomal enzyme induced by acetate.

Since it cannot be ruled out that isocitrate lyase in the heterologous system might be synthesized as a larger precursor and artefactually degraded to the size of the functional subunit, labelling in the reticulocyte lysate was carried out with formyl[^{35}S]methionyl-tRNA which is incorporated only into the amino-

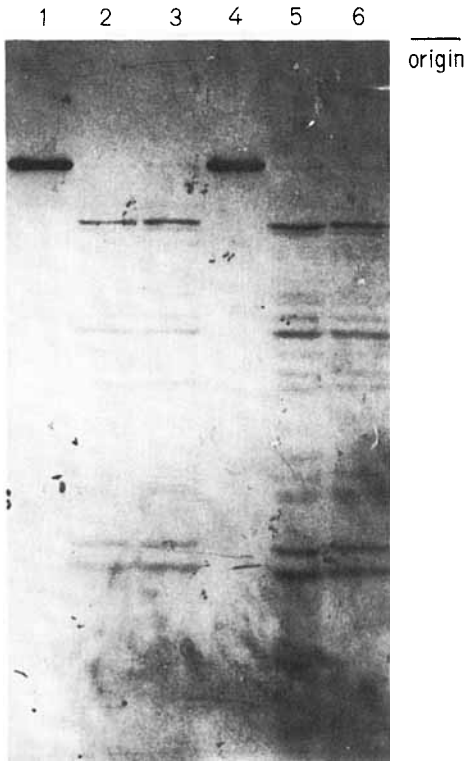

Fig. 3. *Limited proteolysis of isocitrate lyase synthesized* in vivo *and in a reticulocyte lysate.* Isocitrate lyase was immunoprecipitated from cells grown on acetate and [^{35}S]sulfate; isocitrate lyase was also immunoprecipitated from a reticulocyte lysate after incubation with poly(A)-containing RNA from cells grown on acetate and [^{35}S]methionine. The washed immunoprecipitates were dissolved in dodecylsulfate-containing buffer and treated with trypsin [8]. Then the samples were subjected to electrophoresis in the presence of dodecylsulfate and the dried gels were autoradiographed. Lane 1: immunoprecipitate from reticulocyte lysate. Lane 2: immunoprecipitate from reticulocyte lysate, treated with 200 μg/ml trypsin for 5 min at 23 °C. Lane 3: immunoprecipitate from [^{35}S]sulfate-labelled cells, treated with trypsin as in 2. Lane 4: immunoprecipitate from [^{35}S]sulfate-labelled cells. Lane 5: immunoprecipitate from reticulocyte lysate, treated with 200 μg/ml trypsin for 20 min at 23 °C. Lane 6: immunoprecipitate from [^{35}C]sulfate-labelled cells, treated with trypsin as in 5

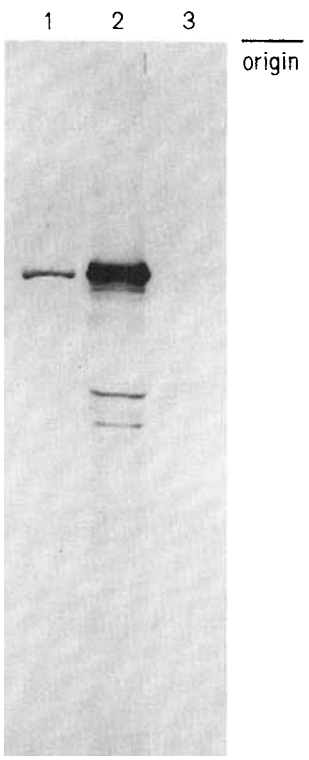

Fig. 4. *Synthesis of isocitrate lyase by free and membrane-bound ribosomes.* Free and membrane-bound ribosomes were prepared from *Neurospora* cells grown on acetate by sucrose density gradient centrifugation as described [43]. Total ribosome fractions obtained from 5 g cells (wet weight) were resuspended in a postribosomal supernatant from a nuclease-treated reticulocyte lysate and incubated for 60 min in the presence of [^{35}S]methionine. Immunoprecipitates were obtained from the reisolated postribosomal supernatants. These were subjected to gel electrophoresis and autoradiography. Lane 1: reference isocitrate lyase, immunoprecipitated from an extract of [^{35}S]sulfate-labelled cells. Lane 2: free ribosome fraction; immunoprecipitation utilizing Sepharose-bound protein A. Lane 3: membrane-bound ribosome fraction; immunoprecipitation as in 2

terminal position and not released by methionyl amino-peptidase [41, 42]. Labelling with this probe thus demonstrates an unchanged amino terminus. Fig. 2 (lane 3) shows that isocitrate lyase becomes labelled with this probe and that the apparent molecular weight is the same as that of the product labelled with methionine. Therefore, the original translation product of isocitrate lyase does not appear to have an amino-terminal extension, at least not of a size commonly found for 'signal sequences' present in many secretory proteins.

In order to identify definitely the product obtained *in vitro*, limited proteolysis of the immunoprecipitate from the reticulocyte lysate was performed. The pattern of labelled tryptic fragments of the protein synthesized *in vivo* is compared to the tryptic pattern obtained with an immunoprecipitate of the authentic labelled protein. Fig. 3 shows these patterns side by side. Clearly, the positions of all bands in the gel coincide which indicates that the products obtained *in vivo* and *in vitro* are identical.

Intracellular Site of Synthesis of Isocitrate Lyase

A cell-free homogenate prepared from *Neurospora* cells grown on acetate carried out synthesis of isocitrate lyase which had the same apparent molecular weight as the authentic protein as well as the product obtained from the heterologous cell-free system *in vitro* (not shown here). In order to identify the type of ribosomes which are active in synthesizing isocitrate lyase, synthesis was performed with isolated free and membrane-bound ribosomes in a read-out system, employing a postribosomal supernatant from a reticulocyte lysate. The two ribosomal fractions were

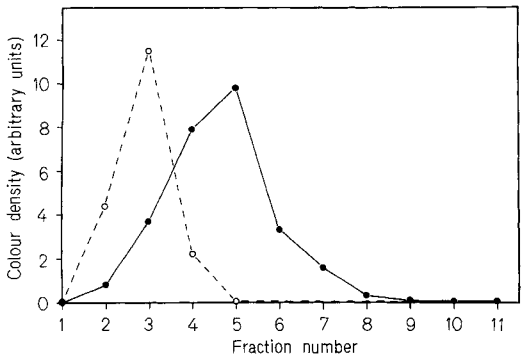

Fig. 5. *Gel filtration on Sephadex G-100 of isocitrate lyase synthesized* in vivo *and* in vitro. A cell-free extract was prepared from *Neurospora* cells grown on acetate in the presence of [^{35}S]sulfate by grinding cells with sand and buffer containing 0.44 M sucrose, 1 mM EDTA and 30 mM Tris/HCl, pH 7.5. A postribosomal supernatant was prepared by centrifugation for 1 h at 166000 × *g*. A reticulocyte lysate was incubated with poly(A)-containing RNA from cells grown on acetate and [^{35}S]methionine for 60 min. The postribosomal supernatant was prepared by centrifugation for 1 h at 166000 × *g*. The two types of supernatant (0.5 ml) were brought to a final concentration of 0.3 M KCl and subjected to gel filtration on Sephadex G-100. The bed of the column (0.9 × 55 cm) was equilibrated with 0.3 M KCl, 10 mM Tris/HCl, pH 7.5 and the applied samples were eluted with the same buffer at a rate of 18 ml/h. Immunoprecipitates were prepared from each fraction and analysed by electrophoresis. The gels were autoradiographed. The exposed film was subjected to densitometry at 578 nm. The area under the peak corresponding to isocitrate lyase was measured. (O---O) Postribosomal supernatant from labelled *Neurospora* cells; (●——●) postribosomal supernatant from reticulocyte lysate. Hemoglobin was eluted from the column in fractions 5–6, cytochrome *c* in fraction 11. Dextran blue appeared in fraction 3

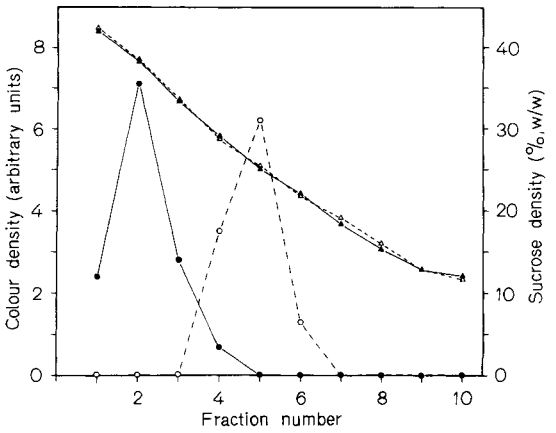

Fig. 6. *Density gradient centrifugation in the absence and presence of detergent of isocitrate lyase synthesized* in vitro. Poly(A)-containing RNA from cells grown on acetate was translated in a reticulocyte lysate in the presence of [^{35}S]methionine for 60 min. The postribosomal supernatant was prepared and made 0.3 M KCl. A portion of 0.5 ml of this supernatant was layered on a linear density gradient containing 0.3 M KCl, 10 mM Tris/HCl, pH 7.5, 5–45% (w/w) sucrose. To a second portion of the supernatant (0.5 ml) Triton X-100 was added to a final concentration of 1% and the mixture was layered on a gradient as above but containing 1% Triton X-100. Both gradients were centrifuged for 60 h at 166000 × *g* in a Beckman ultracentrifuge, rotor 50 Ti. The gradients were fractionated into 1.3-ml portions. Sucrose concentrations were determined in a Zeiss refractometer. Immunoprecipitation employing Sepharose-bound protein A was carried out with the individual fractions. Immunoprecipitates were dissolved in dodecylsulfate-containing medium and analysed by gel electrophoresis. The dried gel slabs were subjected to autoradiography and densitometry at 578 nm. In a parallel experiment, the postribosomal fraction of a cell homogenate obtained from cells grown on acetate and [^{35}S]-sulfate was applied to the same gradients. The gradients were centrifuged and analysed as above. Authentic isocitrate lyase was found in fractions 1 and 2 in both Triton-containing and Triton-free gradients. Hemoglobin was found in fraction 2 in absence and presence of Triton. (●——●) Gradient without Triton; (O---O) gradient with Triton; (△, ▲) sucrose concentration; (○, ●) colour density

prepared by density gradient centrifugation. Total ribosomal fractions obtained from 5 g cells (wet weight) were incubated with a postribosomal supernatant from a reticulocyte lysate. Only utilizing free ribosomes, isocitrate lyase was found among the products *in vitro* (Fig. 4).

Properties of Isocitrate Lyase Synthesized in Reticulocyte Lysates

Protein synthesis was carried out in a reticulocyte lysate in the presence of [^{35}S]methionine programmed with *Neurospora* mRNA and a postribosomal supernatant was prepared by centrifugation. The resultant supernatant was subjected to gel filtration on Sephadex G-100. In a parallel run, a postmitochondrial supernatant was analysed which was prepared from *Neurospora* cells grown in the presence of acetate and [^{35}S]-sulfate. Isocitrate lyase was immunoprecipitated from fractions eluted from the bed. The authentic enzyme is eluted with the void volume (Fig. 5). When Sephadex G-200 was employed the eluted enzyme had a molecular weight of about 280000, in good agreement with the molecular weight determined in studies in which the enzymatic activity was used to detect the enzyme [27]. Obviously, the functional enzyme is a tetramer. In contrast, isocitrate lyase synthesized *in vitro* is eluted from Sephadex G-100 (Fig. 5) and G-200 (not shown) with a molecular weight corresponding to that of hemoglobin. This finding indicates that the product *in vitro* is present as a monomer.

The supernatant of the reticulocyte lysate was also subjected to sucrose density gradient centrifugation. Isocitrate lyase synthesized *in vitro* sediments like hemoglobin (Fig. 6).

When gel filtration and gradient centrifugation are carried out in the presence of 1% Triton, the functional protein does not show any change in its molecular weight or density. Isocitrate lyase synthesized *in vitro*, however, displays a lower density.

These findings suggest that the newly synthesized isocitrate layse occurs in a soluble form in the cytosol

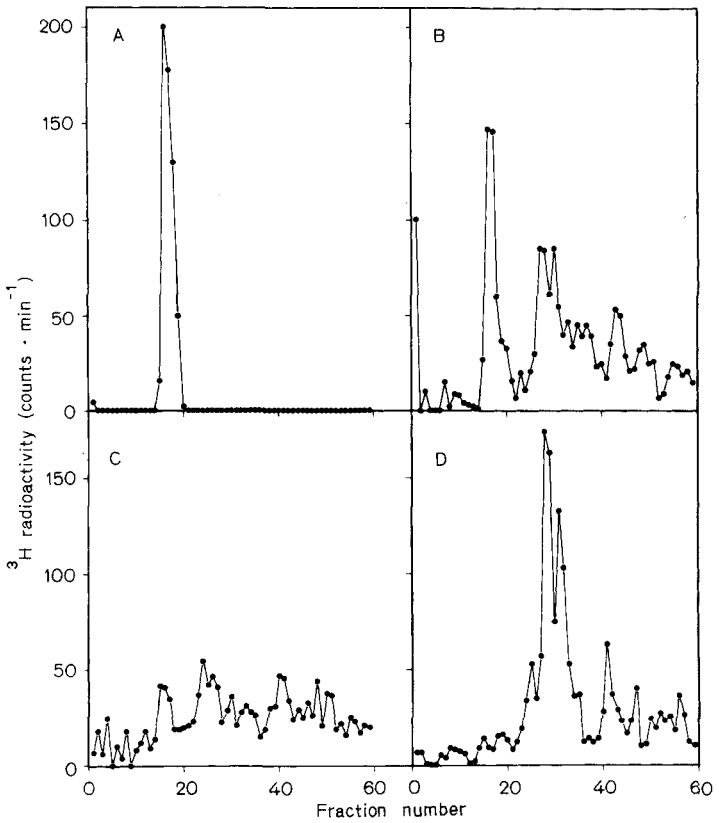

Fig. 7. *Effect of proteinase K treatment of a* Neurospora *particle fraction on isocitrate lyase.* Cells grown on acetate and [^{3}H]leucine were broken by grinding with sand and sucrose medium containing 0.44 M sucrose, 1 mM EDTA, 30 mM Tris/HCl, pH 7.5. Unbroken cells and cell walls were removed by centrifugation twice for 5 min at 5000 × *g*. Then a 12-min 17300 × *g* centrifugation was carried out (the pellet was resuspended and recentrifuged in a low and high spin under the same conditions). This pellet was resuspended in sucrose medium and divided into four portions. These portions were treated as follows: (A) no further treatment; (B) incubation with proteinase K for 60 min at 4 °C (100 µg/ml), addition of 0.1 mM phenylmethylsulfonyl fluoride; (C) sonication for 5 min (Branson sonifier, microtip), then proteinase K treatment as in B; (D) addition of sodium deoxycholate to a final concentration of 1%, then proteinase K treatment as in B. To samples A – C Triton X-100 was added (final concentration 1%) and with samples A – D immunoprecipitation was carried out. Immunoprecipitates were analysed by electrophoresis in the presence of dodecylsulfate on horizontal slab gels [7]. The gels were cut into 1-mm slices and radioactivity was determined. Origin is fraction 1

fraction; it is present as a monomer, and it binds Triton, thus exhibiting an amphipathic character. In contrast, the functional protein is a tetramer which is unable to bind detergent.

Transfer of Isocitrate Lyase into Glyoxysomes in vitro

The question whether isocitrate lyase synthesized in cell-free systems can be transferred into glyoxysomes by a post-translational mechanism, cannot be answered easily and directly. Isolation of a pure glyoxysome preparation has not been achieved with *Neurospora* wild type and is very difficult with other organisms. It has been shown that upon sucrose density gradient centrifugation of *Neurospora* particle preparations, the bands containing mitochondria and glyoxysomes overlap [21, 22]. Furthermore, *Neurospora* glyoxysomes are labile organelles and with each step of purification a considerable part of them becomes destroyed [21, 22]. Therefore, we have not attempted to employ purified glyoxysomes to demonstrate transfer *in vitro*, but instead we have used an indirect approach. Since in a non-induced state glyoxysomes are virtually absent from *Neurospora* particle preparations, these can serve as a control which should not take up isocitrate lyase *in vitro*. Particle preparations from induced cells should take up the extraglyoxysomal precursor form. In such particle preparations unspecific binding is a major source of error. Therefore, resistance of intraglyoxysomal isocitrate lyase to added proteinase was employed as a criterion for transmembrane translocation. *Neurospora* cells grown on acetate were labelled with [^{35}S]sulfate. Cells were harvested, broken by sand grinding in sucrose medium and a 15-min 17300 × *g* pellet was prepared. This pellet contained 25% of total isocitrate lyase. The pellet was resuspended in sucrose-containing buffer and treated with proteinase K under the following conditions: (a) without further treatment; (b) after addition of deoxycholate to a final concentration of 1%; and (c) after sonication. After inhibition of proteinase K with phenylmethylsulfonyl fluoride and lysis of the

samples with Triton, isocitrate lyase was immunoprecipitated. Fig. 7 shows that part of isocitrate lyase in the control sample is resistant to proteinase K (about 50%), whereas after treatment with deoxycholate or after sonication the enzyme was digested so that it could not be immunoprecipitated. Resistance to proteinase K therefore appears to be a reliable criterion for isocitrate lyase being enclosed by a membrane.

For transfer experiments *in vitro*, a reticulocyte lysate was programmed with poly(A)-containing RNA from *Neurospora* cells grown on acetate. [^{35}S]Methionine was incorporated and a post-ribosomal supernatant was prepared. Simultaneously, 12-min 17000 $\times g$ pellet fractions were prepared from cells grown on sucrose and from cells grown on acetate. Samples of the reticulocyte supernatant were resuspended with the two different particle preparations and the suspensions incubated for 60 min. Then the particles and supernatants were separated again and isocitrate lyase was immunoprecipitated from the fractions. Immunoprecipitates were analysed by gel electrophoresis, stained with Coomassie blue and subjected to autoradiography (Fig. 8). The stained gels show in the case of acetate-induced cells that part of the isocitrate lyase is released from the glyoxysomes during incubation with reticulocyte lysate and appears in the supernatant (about 50% of the input). This indicates that the transfer system *in vitro* is very labile. The autoradiographs show that isocitrate lyase synthesized *in vitro* does not adsorb unspecifically to cell organelles, since no isocitrate lyase was found in the pellet fraction which was obtained from cells grown on sucrose. Rather, it is recovered in the supernatant. In contrast, part of the isocitrate lyase becomes associated with the particle preparation from acetate-induced cells. Treatment of such a particle fraction with proteinase K does not result in complete digestion of the isocitrate lyase synthesized *in vitro*. In connection with the experiments designed to show proteinase resistance, it is suggested that part of the added isocitrate lyase is transferred into the glyoxysomes.

There is no direct evidence that the subunits are assembled to the functional enzyme within the glyoxysome. However, this is the most probable mechanism. The transfer reaction *in vitro* takes place in the presence of relatively large amounts of pre-existent assembled enzyme which has leaked out from the organelles. If the assembly occurred outside the glyoxysomes, then the pre-existent enzyme should behave as the newly synthesized radioactive protein. This is obviously not the case.

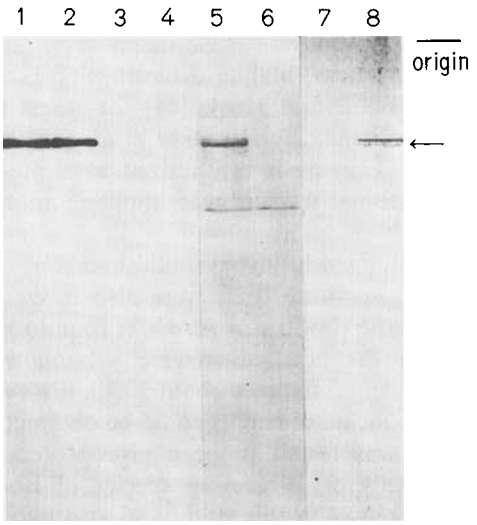

Fig. 8. *Transfer of isocitrate lyase* in vitro. Poly(A)-containing RNA from cells grown on acetate was translated in a reticulocyte lysate for 60 min in the presence of [^{35}S]methionine. The postribosomal supernatant was prepared and mixed with a 12-min 17000 $\times g$ particle fraction, which was prepared from cells grown either on sucrose or acetate. After mixing, one half was withdrawn and kept at 0 °C, the second half was incubated at 25 °C for 60 min. From all portions, particles and supernatant were then separated again by centrifugaton for 12 min at 17000 $\times g$. Triton was added to the supernatant (final concentration 1%) and 5 µg isolated isocitrate lyase was added as carrier. The pellets were taken up in 0.44 M sucrose, 1 mM EDTA, 10 mM Tris/HCl, pH 7.5 and treated with proteinase K as described in the legend to Fig. 7, and then Triton was added. Direct immunoprecipitation was carried out with all fractions and immunoprecipitates were analysed 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: supernatant; 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. Arrow indicates position of protein stain of isocitrate lyase

DISCUSSION

The findings presented in this paper support the existence of a post-translational mechanism for the transfer of isocitrate lyase into glyoxysomes. The following concept of the intracellular tranfer of this enzyme can be proposed. The enzyme subunits are released from free ribosomes into an extraglyoxysomal precursor pool in the cytosolic compartment. They are translocated across the glyoxysomal membrane. Assembly to the functional enzyme may then occur in the glyoxysomes. The data do not agree with a co-translational transfer of isocitrate lyase, viz. that this enzyme is synthesized on ribosomes attached to the endoplasmic reticulum, vectorially discharged into the lumen and then transferred to nascent glyoxysomes before these are budded off. It should be noted that the mechanism proposed here does not necessarily apply to other glyoxysomal proteins. In particular, it must be considered that glyoxysomal proteins with attached carbohydrate residues pass through the Golgi complex before they reach the glyoxysome.

The primary translation product of isocitrate lyase in the reticulocyte lysate had the same apparent molec-

ular weight as the subunit of the functional enzyme. In this respect, isocitrate lyase differs from another glyoxysomal protein, malate dehydrogenase, as first reported by Walk and Hock [44] for water melon seeds and confirmed by Riezman et al. [38] for cucumber. This enzyme is synthesized as a precursor with an additional sequence of apparent molecular weight 5000.

In the latter study glyoxysomal catalase, malate synthase and isocitrate lyase were also investigated. For catalase, the product *in vitro* was found to differ slightly from the labelled enzyme subunit *in vivo* (molecular weight difference about 1000). With malate synthase no conclusive results could be obtained since this enzyme was found to be a glycoprotein. Two immunologically cross-reacting species of isocitrate lyase were detected in this study and also two translation products *in vitro*, of which at least one was smaller than the labelled product *in vivo*. Since the relationship between translation *in vivo* and *in vitro* was unclear, no difinite conclusion could be made on the size of the precursor. Furthermore, a possible processing of the products *in vitro* by the wheat germ extract was not excluded.

Taken together, the available data show that proteolytic processing of an additional sequence is not an essential event in membrane translocation with glyoxysomes. Furthermore, when cleavage of a larger precursor is involved, it has not been shown that the additional sequence has a 'signal' function. In this respect post-translational transfer into glyoxysomes resembles that into mitochondria, where precursors with and without additional sequences were described [8–10, 12, 43, 45, 46].

The ability of the isocitrate lyase subunit, synthesized *in vitro*, to bind detergent may reflect a significant feature of precursors of proteins which must be translocated across membranes or integrated into membranes (cf. [8]). For malate dehydrogenase which is transferred into the mitochondrial matrix it was found that the monomer but not the dimer interacts with phospholipid vesicles [47]. Detergent binding has also been reported for the water-soluble form of the toxic peptide melittin from bee venom which can insert into membranes [48] or for the soluble precursor form of M13 coliphage coat protein [49].

A difference in conformation between precursor and subunit of isocitrate lyase could be the reason why precursor molecules do not assemble outside the organelles. Other reasons, however, such as modification or binding of lipids, could also be responsible.

An unambiguous demonstration and analysis of the transfer of proteins into glyoxysomes in reconstituted systems *in vitro* is very difficult to achieve. Nevertheless, convincing demonstration of transfer of extremely small amounts of polypeptides synthesized *in vitro* continues to be a problem with other cell organelles which can be obtained in higher purity. The approach which we have used in this study, namely to compare transfer into a particle fraction from acetate-induced cells which contain glyoxysomes to that from non-induced cells, is a first step towards a more detailed analysis.

Recently, Kindl et al. [50] have reported that the cytosolic fraction of castor been seedlings after pulse labelling with radioactive amino acids *in vivo* contains the glyoxysomal enzymes malate synthase, catalase and isocitrate lyase, all of which showed labelling kinetics of precursor molecules. Although the molecular weights were not determined, these findings are in line with the data presented here.

A number of similarities exist between the results described in this paper and results obtained on the biogenesis of rat liver peroxisomal catalase. It has been shown by Lazarow and de Duve several years ago by labelling studies *in vivo* that catalase passes through a cytosolic precursor pool as an apomonomer [13, 14]. These precursor molecules were found to be synthesized by free ribosomes [16]. No additional sequence was detected in the translation product of rat liver mRNA in heterologous systems [15, 16]. However, in these studies the existence of a cleaved 'signal sequence' was not completely ruled out, and it was actually suggested that such a hypothetical sequence was lost by artificial processing [16]. Interestingly, another peroxisomal enzyme, uricase, was also found to be synthesized *in vitro* without an additional sequence [16].

The authors are grateful to H. Bliedung, S. Krull and S. Pitzel for skilful technical assistence. This work was supported by the *Deutsche Forschungsgemeinschaft* Ne 101/17.

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