Distribution of Isocitrate Lyase in Induced and De-adapting Cells of the Green Alga *Chlorella fusca*

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(Received 14 April 1987; revised 15 June 1987)

Colloidal gold labelled goat anti-rabbit IgG has been used in conjunction with a rabbit anti-isocitrate lyase antibody, to locate isocitrate lyase in the green alga *Chlorella fusca*. Under induced conditions isocitrate lyase was apparently uniformly distributed in the cytoplasm. Under de-adapting conditions (when enzyme activity had diminished by 45% from the fully induced level), the amount of gold label bound to sections was proportionately reduced although its cytoplasmic location was unchanged. Immunodetection of protein transferred to nitrocellulose after electrophoresis confirmed that there was loss of isocitrate lyase protein during de-adaptation.

INTRODUCTION

The glyoxylate bypass to the tricarboxylic acid cycle, which utilizes the enzymes isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), is commonly found serving one of two metabolic functions. In plant seeds that contain stored fats the bypass functions during early seedling development to convert stored fat to sugar for dissemination through the tissues of the developing seedling. For this function a cellular organelle – the glyoxysome – is formed, which contains both the enzymic machinery for fatty acid oxidation to acetyl units and the glyoxylate bypass enzymes (Tolbert & Essner, 1981; Cioni *et al.*, 1981; Trelease, 1984). A related metabolic function has been shown in some invertebrates (Trelease, 1984).

In micro-organisms, the glyoxylate bypass is commonly used for the aerobic metabolism of an externally supplied two-carbon growth substrate such as acetate or ethanol (Kornberg, 1966). Here the bypass functions as a classical anaplerotic pathway allowing assimilation of carbon into metabolic intermediates which are required for biosynthesis, but are not formed directly by the pathways of oxidative energy generation (Kornberg, 1966). In micro-organisms the location of isocitrate lyase and malate synthase is variable. Isocitrate lyase is a soluble cytoplasmic enzyme in those bacteria that grow aerobically on acetate, such as *Escherichia coli* and *Pseudomonas indigofera* (Cioni *et al.*, 1981). Glyoxysomes (i.e. microbodies containing isocitrate lyase, but not necessarily the enzymes of fatty acid oxidation) have been found in *Tetrahymena*, *Saccharomyces* and *Neurospora* (Tolbert & Essner, 1981) and more recently in *Candida* and *Hansenula* species (Zwart *et al.*, 1983). Amongst the algae, *Euglena* is known to form glyoxysomes (Horrum & Schwartzbach, 1981), but no clear evidence for the cellular location of the glyoxylate bypass enzymes has been documented for the green algae such as *Chlorella* and *Chlamydomonas*.

When acetate-adapted *Chlorella fusca* cultures are transferred to glucose-containing media, the activity of isocitrate lyase declines to a negligible level in 25–30 h, and this correlates with a loss of the enzyme protein from the 100 000 g supernatant fraction of cell extracts (John *et al.*, 1970; Henley & Thurston, 1986). This disappearance of the enzyme protein could be due to proteolytic degradation, but could equally result from binding of (inactive) enzyme to a membrane fraction or from inactive enzyme molecules forming aggregates which would sediment at 100 000 g. We have therefore studied the cellular location of isocitrate lyase in *C. fusca* in order to establish the site of glyoxylate bypass function for this organism, but also

to establish whether the 'disappearance' of isocitrate lyase could be attributed to relocation within the cell.

METHODS

Culture of organism. *Chlorella fusca* Cambridge Culture Collection of Algae and Protozoa strain 211/8p was grown autotrophically at 25 °C as described by John *et al.* (1970). Isocitrate lyase was induced (to its maximum specific activity) by 24 h incubation in the dark after addition of 25 mM-sodium acetate. Disappearance of isocitrate lyase activity was initiated by addition of 27 mM-glucose to acetate-adapted cultures that had been washed twice and resuspended in nitrogen-free medium and returned to the dark at 25 °C (John *et al.*, 1970).

Anti-isocitrate lyase IgG. Anti-isocitrate lyase antibodies were raised in rabbits. Serum was fractionated by ammonium sulphate precipitation and affinity chromatography on staphylococcal protein A-Sepharose (Pharmacia), as described previously (Henley & Thurston, 1986). About 4% of the resulting IgG fraction bound to isocitrate lyase when a constant amount of enzyme was challenged with varying amounts of IgG.

Preparation of cell extracts. Samples of culture (100 ml) were harvested, washed twice in 50 ml 0.1 M-potassium phosphate, 5 mM-DTT, 1 mM-EDTA, pH 6.7, and resuspended in 2 ml of the same solution. The washed, concentrated samples were stored at -70 °C overnight. Samples were allowed to thaw at room temperature until the residual ice could be melted by vortex mixing, after which each sample was passed twice through an ice-cold French pressure cell at 135 MPa. The crude broken cell suspensions (>97% cell breakage) were divided; 4 × 150 µl were immediately extracted and precipitated by the chloroform/methanol method of Wessel & Flügge (1984) and the remainder was centrifuged at 200 000 g for 30 min at 4 °C. The supernatant was stored on ice whilst isocitrate lyase activity was assayed (using 2–5 µl amounts), after which 4 × 150 µl samples were subjected to chloroform/methanol precipitation as above. Protein concentration was determined on both supernatants and chloroform/methanol precipitated samples by the method of Hess *et al.* (1978), using bovine plasma albumin as standard, after first dissolving the precipitates in 0.1 M-NaOH. Centrifugation at 200 000 g for 30 min completely removed green membrane material from the supernatant cell extracts as previously achieved by centrifugation at 100 000 g or 120 000 g for longer periods (John *et al.*, 1970; Thurston *et al.*, 1973).

Isocitrate lyase assay. Activity of the enzyme was measured at 30 °C by the continuous spectrophotometric method of Kornberg (1965). Cuvettes contained 990 µl assay mix composed of: 100 µmol imidazole.HCl pH 6.8, 5 µmol MgCl₂, 1 µmol DTT, 10 µmol phenylhydrazinium chloride and 10 µmol trisodium isocitrate. Assay mix was made from stock solutions held on ice. DTT and phenylhydrazinium chloride solutions were made up on the day of use. Cuvettes were equilibrated to the assay temperature for 3 min before the sample cuvette was taken out for addition of 5–10 µl of suitably diluted cell-extract. The cuvette was quickly inverted several times to mix the contents and returned to the spectrophotometer (Varian DMS90). Change in absorbance at 324 nm relative to the reagent blank was recorded for about 5 min. Sodium glyoxylate standards were used to derive activity as µmol glyoxylate formed min⁻¹.

Electrophoresis and immunoblotting. Chloroform/methanol precipitated samples were dissolved in loading buffer, boiled for 5 min and separated by electrophoresis on 10% (w/v) acrylamide slab gels as described by Laemmli (1970). Total cell extract samples were centrifuged briefly before loading to remove insoluble cell-wall material. After electrophoresis, gels were cut in half so that parallel samples could be either stained for total protein with Coomassie brilliant blue or transferred to nitrocellulose for immunoblotting. Proteins were electroblotted to nitrocellulose and antibody-labelled using the conditions described by Zamanian & Mason (1987). Anti-isocitrate lyase was used at 5 µg ml⁻¹ (1:1000). The second antibody was affinity purified goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) at a dilution of 1:2000. After colour development, the filters were dried and soaked in glycerol to allow transmission densitometry. The peaks due to isocitrate lyase bands were cut out and weighed for comparison.

Electron microscopy. The procedure broadly followed Beesley *et al.* (1984). Cells were pelleted from 1 ml amounts of culture by 10–15 s centrifugation in a microcentrifuge and fixed by resuspension with 1 ml 1% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate, pH 7.2. Excess glutaraldehyde was removed by washing in 0.1 M-sodium cacodylate, pH 7.2. The cells were dehydrated by 10 min periods in 50, 70, 90 and 100% (v/v) ethanol. The 100% ethanol was replaced twice. The dehydrated cells were embedded in LR White Resin (London Resin, Basingstoke, Hants, UK: four changes each of 1.5 h) polymerized by 20 h treatment at 60 °C in air-tight gelatin capsules.

Sections on nickel grids were incubated at room temperature for all steps, using two drops of each reagent successively. The incubations were: 0.05 M-Tris/HCl, 0.01% Tween 20, 0.1% gelatin, 1.0% (w/v) ovalbumin, pH 7.4 (TTGO buffer) for 4 min; TTGO buffer containing 50 µg anti-isocitrate lyase IgG ml⁻¹ (1:100) for 1 h; TTGO buffer, five times for 1 min each; colloidal gold labelled goat anti-rabbit IgG (Auroprobe EM GAR G10, Jensen Pharmaceutica) diluted 1:4 (v/v) with TTGO buffer for 1 h; tap water, five times for 1 min each. Gold-labelled sections were stained with 2% (w/v) uranyl acetate and Reynold's lead citrate, each for 5 min. The concentration of primary antibody was optimized in preliminary experiments. Sections were examined in a JEOL 100CX Mk 2 microscope.

Fig. 1. Anti-isocitrate lyase dependent immunogold labelling of sections of *C. fusca*. (a) Part of an autotrophically grown cell showing the background level of gold-labelling. (b) Part of a cell fully induced for isocitrate lyase. (c) One unlabelled (A) and one heavily labelled (B) cell from a sample where equal numbers of autotrophic and acetate-adapted cells were mixed immediately before fixation. (d) Part of a cell sampled from de-adaptive conditions. The culture had been incubated in nitrogen-free medium containing glucose for 15.5 h, during which isocitrate lyase activity had declined by 45%. Bars 1 μ m.

Fig. 2. Electrophoretic separation on a 10% polyacrylamide-SDS gel of total protein (tracks 1–3) and 200 000 *g* supernatant protein (tracks 4–6) of samples from three *C. fusca* cultures. (a) Coomassie blue stained protein; (b) immunodetection of isocitrate lyase after blotting onto nitrocellulose. Tracks 1 and 4 are extracts from an autotrophic culture, tracks 2 and 5 are extracts from a fully induced culture and tracks 3 and 6 are extracts from a de-adapting culture (see legend to Fig. 1d). Isocitrate lyase specific activity in the 200 000 *g* supernatant extracts was [$\mu\text{mol glyoxylate formed min}^{-1} (\text{mg protein})^{-1}$]: autotrophic, $\ll 0.023$ (assay detection limit); induced, 48.9; de-adapting, 26.9. Tracks 1–3 were loaded with 40 μg protein and tracks 4–6 with 20 μg protein. The arrow marks the isocitrate lyase band, located from the position of pure enzyme run in a parallel track. The nitrocellulose sheet photographed in (b) showed bands in tracks 3 and 6 with 60% and 61% of the intensity of staining in tracks 2 and 5 respectively, by densitometry as described in Methods.

RESULTS

Immunocytochemical localization of isocitrate lyase

Anti-isocitrate lyase-immunogold labelling was applied to thin sections from three *C. fusca* cultures. (1) A culture growing exponentially in the light acted as a negative control (Fig. 1a), as under these conditions of growth the amount of isocitrate lyase was only 1–2 molecules per cell

(Thurston, 1977) and sections showed only background labelling with gold particles. (2) Fully induced cultures, on the other hand, contained about 2×10^6 molecules of isocitrate lyase per cell (Thurston, 1977) and heavy labelling with gold particles was observed on sections of these cells (Fig. 1*b*), with the gold particles mostly over areas of cytoplasm. Gold labelling was not apparently associated with any microbody- or glyoxysome-like structure, nor were the chloroplast or mitochondria more labelled than non-specific background. In order to eliminate any possible difference of treatment during sample preparation, a mixture of the two cultures was processed as a single sample (Fig. 1*c*). The gold labelling of cells in sections from this sample was clearly differentiated, cells resembling either one or other of the separately processed samples. Specificity of labelling was further verified by showing only background levels of labelling when no primary antibody was included and when anti-isocitrate lyase IgG was replaced by an unrelated IgG preparation (containing antibody to a benzene dioxygenase polypeptide from *Pseudomonas putida*, Zamanian & Mason, 1987). (3) The third culture from which samples were sectioned and immuno-gold labelled had been induced for isocitrate lyase and transferred to de-adapting conditions for 15.5 h, during which time isocitrate lyase activity had declined by 45%. Gold particles were distributed over cytoplasmic areas of these sections (Fig. 1*d*); fewer gold particles were found than on sections of induced cells (Fig. 1*b*) and much of the gold was in clumps.

Electrophoresis and immunoblotting

After lipid extraction of chloroform/methanol precipitation (Wessel & Flügge, 1984), total protein from broken cell suspensions can be separated by electrophoresis under denaturing conditions. We compared the protein in total extracts with that in 200 000 *g* supernatants (Fig. 2*a*). The band due to isocitrate lyase was clearly visible in both types of sample from both induced and de-adapting cultures. Most particularly, the total protein samples (lanes 1–3) showed the isocitrate lyase band as less intense in the de-adapting sample (lane 3) than in the induced sample (lane 2) to an extent which cannot be distinguished from the same comparison for 200 000 *g* supernatant samples (cf. lanes 5 and 6). The disappearance of isocitrate lyase protein from supernatant extracts was not therefore due to membrane-adsorption or precipitation of inactive isocitrate lyase, but must be due to proteolytic degradation as we have previously proposed (John *et al.*, 1970; Thurston *et al.*, 1973; Henley & Thurston, 1986). The immunoblotting result (Fig. 2*b*) is consistent with the above, as for both total and supernatant extracts there was a similar diminution of isocitrate lyase (as indirect antibody staining) comparing induced and de-adapting samples. Densitometric estimation of the relative amount in each stained band gave the de-adapting sample to be 60% of the induced sample from analysis of total cell-extracts (lanes 2 and 3) and 61% for supernatant extract samples (lanes 5 and 6). Immunoblotting also showed that no significant amount of isocitrate lyase antigen of altered size was present in the de-adapting sample.

DISCUSSION

From the distribution of isocitrate lyase revealed by immunogold labelling of sections of acetate-adapted *C. fusca* cells, it is most unlikely that glyoxylate bypass enzymes are associated with any glyoxysome-like structure in this organism. Our data suggest, rather, that isocitrate lyase is a cytosolic protein. It should at the same time be noted that this strain is one of a small group in which isocitrate lyase may be induced to a quite remarkably high level ($\sim 7\%$ of soluble protein; John & Syrett, 1968) and it may be that compartmentalizing isocitrate lyase into glyoxysomes confers no advantage when so much of it is present. Equally it may be that strains unable, for one reason or another, to make glyoxysomes are only able to grow on acetate with a relatively enormous amount of isocitrate lyase activity.

The disappearance of isocitrate lyase activity upon addition of glucose has been correlated with disappearance of isocitrate lyase protein from 100 000 *g* supernatant extracts (John *et al.*, 1970). We recently confirmed, using direct antibody precipitation, that such supernatant extracts become depleted of isocitrate lyase protein, but it was impossible to distinguish between

proteolytic degradation and protein relocation (Henley & Thurston, 1986). Specifically, if a part of the isocitrate lyase inactivation process involved binding to a membrane or aggregation to form a protein precipitate (Prouty & Goldberg, 1972), this would result in loss of enzyme protein from 100 000 g supernatant extracts. The data presented here show that such processes do not contribute significantly to isocitrate lyase protein disappearance, which must therefore be largely, if not exclusively, due to proteolytic degradation. Although isocitrate lyase does not form precipitable aggregates during de-adaptation, from Fig. 1(d) it appears that the enzyme is markedly more aggregated than under inducing conditions, but the reliability and significance of this observation must await further study.

We are very grateful to Jeremy Mason and Mariam Zamanian for the gift of anti-benzene dioxygenase A1 subunit IgG and for their advice and help with immunoblotting. We are also very grateful to Tricia Dopping-Hepenstal and Julian Beesley for advice on immunogold labelling.

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