The Subcellular Localization of Glyoxylate Cycle Enzymes in *Coprinus lagopus* (sensu Buller)

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(Received 10 November 1972)

SUMMARY

The derepression and localization of the glyoxylate cycle enzymes in *Coprinus lagopus* (sensu Buller) have been studied. Differential and sucrose density gradient centrifugation of extracts indicated a particulate localization for isocitrate lyase (EC. 4.1.3.1) and malate synthase (EC. 4.1.3.2). The particle is distinct from, and less dense than, the mitochondrion and is considered to be a glyoxysome.

INTRODUCTION

Anaplerotic metabolic pathways, such as the glyoxylate cycle, effect the conversion of growth substrate into cell material when micro-organisms are grown with C₂ compounds as the sole source of carbon (Kornberg, 1966). Whilst the glyoxylate cycle has a number of reactions in common with the tricarboxylic acid cycle, there is considerable evidence that these two cycles are spatially separated in the eukaryotic cell. For example, in *Tetrahymena pyriformis* (Müller, Hogg & De Duve, 1968), *Neurospora crassa* (Kobr, Vanderhaeghe & Combépine, 1969), yeast (Szabo & Avers, 1969) and *Ricinus communis* endosperm (Breidenbach, Kahn & Beevers, 1968; Cooper & Beevers, 1969) at least some of the enzymes of the glyoxylate cycle are located in an organelle separable from the mitochondrion and variously called a glyoxysome or a peroxisome (De Duve, 1969; Avers, 1971; Tolbert, 1971). This spatial separation may provide a basis for the regulation of the activities of the two pathways (Beevers, 1969). The localization of some glyoxylate-cycle enzymes in the basidiomycete *Coprinus lagopus* is discussed in this paper.

METHODS

Growth of fungus and preparation of extracts. H9, a wild-type monokaryotic strain of *Coprinus lagopus* (sensu Buller), was grown in a medium, buffered at pH 6.5 with 0.05 M-potassium phosphate, containing 111 mM-glucose, 37.5 mM-(NH₄)₂SO₄, 295 µM thiamine, and trace elements (Fries, 1953). The buffer, glucose and trace element solutions were autoclaved separately. Oidia were inoculated into 250 ml portions of the medium in 2.3 l penicillin flasks (Jencons Ltd, Hemel Hempstead) to a final concentration of about 10⁶/ml. Cultures were incubated on a rotary shaker (80 rev./min) at 25 °C. Actively growing cultures in glucose medium were harvested by centrifugation, washed in saline, (0.9 %, w/v) and inoculated into fresh medium containing 242 mM-sodium acetate (Hopkin and Williams A. R. grade) in place of glucose. Mycelial samples were stored at -20 °C after harvesting until required.

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Extracts were prepared by blending mycelium in a Virtis Hi-speed 45 Homogenizer (Gardiner, New York, U.S.A.) for two periods of 1 min each at 5 °C. The extraction medium was 0.2 M-potassium phosphate, pH 7.0, when estimation of total mycelial enzyme activity was undertaken, whilst a sucrose (0.25 M), Na₂ EDTA (0.005 M), bovine serum albumin (0.15 %, w/v) medium, pH 7.0, was used when localization studies were carried out (Hall & Greenawalt, 1967).

Fractionation of extracts. Crude mycelial homogenates were subjected to two preliminary centrifugations at 1000g for 10 min (Hall & Greenawalt, 1967) yielding a supernatant (S₁) and a particulate fraction (P₁). S₁ was then centrifuged at 10000g for 30 min resulting in a crude mitochondrial fraction (P₂) and a supernatant (S₂). S₂ was recentrifuged at 35000g for 30 min giving rise to a pellet (P₃) and a supernatant (S₃). Suspensions of P₂ and P₃ in 0.25 M-sucrose were combined for fractionation by sucrose density gradient centrifugation. Discontinuous sucrose gradients (60 to 20 %; 2.26 to 0.63 M) were prepared manually and allowed to stand overnight at 5 °C after which the gradients were approximately linear, as assessed by refractive index measurements. Samples (2 ml) were layered onto the top of the gradient to give a total volume of 20 ml. Centrifugation for 90 min was carried out in a 3 × 23 ml swing-out rotor, by means of an MSE Automatic Superspeed 50 Ultracentrifuge at 30000 rev./min (65000/120000g). The gradients were sampled in 1 ml fractions from the base of the centrifuge tube.

Enzyme assays. Isocitrate lyase (EC. 4.1.3.1) was assayed by the spectrophotometric method of Kornberg (1965); one enzyme unit was defined as giving rise to 1 nmol glyoxylate/min under the conditions of the assay. Malate synthase (EC. 4.1.3.2) was assayed colorimetrically (Flavell & Fincham, 1968); one enzyme unit was defined as giving rise to 1 nmol coenzyme A/min. Malate dehydrogenase (EC. 1.1.1.37) was assayed by the method of Ochoa (1955); one enzyme unit being that amount which oxidized 1 μmol NADH/min. Cytochrome oxidase was assayed by the method of Smith (1955) and enzyme activity expressed as *K* values. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Specific activities are expressed as enzyme units/mg protein.

RESULTS

Derepression of glyoxylate cycle enzymes

Glucose-grown mycelium was harvested, washed and resuspended in a medium containing acetate as sole carbon source. Mycelial samples were extracted and the specific activities of isocitrate lyase, malate synthase and malate dehydrogenase were determined. Isocitrate lyase specific activity reached a maximum some 12 h after transfer of the mycelium to acetate medium, while the other two enzymes continued to increase in specific activity beyond this time, although at a slower rate. There was approximately an eightfold increase in isocitrate lyase and malate synthase specific activities after 12 h, when compared to the repressed level, while that of malate dehydrogenase had increased about thirty times.

Differential centrifugation

Extracts of mycelium that had been transferred to acetate medium were fractionated by the methods outlined and the specific activities of isocitrate lyase, malate synthase, malate dehydrogenase and cytochrome oxidase determined in each of the pellet and supernatant fractions. The results showed that isocitrate lyase, cytochrome oxidase and, to a lesser extent, malate synthase tended to sediment (Table 1). Cytochrome oxidase, which is regarded as an exclusively mitochondrial enzyme (Roodyn, 1967), was found chiefly in P₂ which,

Fig. 1. Sucrose density gradient centrifugation of the 10000 to 35000g particulate fraction from an extract of *Coprinus lagopus*. Distribution of protein (▲), isocitrate lyase (○), malate synthase (●) and cytochrome oxidase (△).

Table 1. *Differential centrifugation of an extract of Coprinus lagopus*

Mycelium transferred to acetate medium was extracted in 0.25 M-sucrose, containing 0.005 M-EDTA and 0.15 % (w/v) bovine serum albumin, pH 7.0. The specific activities of the enzymes were measured in the supernatants and sediments from three centrifugation procedures as indicated.

	Isocitrate lyase	Malate synthase	Malate dehydrogenase	Cytochrome oxidase
1000g × 10 min				
Supernatant (S1)	39	255	4.9	0.048
Sediment (P1)	56	30	2.3	0.043
10000g × 30 min				
Supernatant (S2)	38	189	4.6	0.03
Sediment (P2)	153	84	0.6	0.184
35000g × 30 min				
Supernatant (S3)	42	159	5.0	0.009
Sediment (P3)	380	160	0.6	0.046

therefore, contained most of the mitochondria. The soluble cytochrome oxidase could be due to breakage of mitochondria during the extraction.

Malate dehydrogenase was present in P1 but was largely absent from P2 and P3. This distribution was also found in extracts of repressed (glucose-grown) mycelium where the level of sedimentable malate dehydrogenase activity was similar. The increased malate dehydrogenase of derepressed cells was found in the supernatant fractions (Table 1). Most of the sedimentable isocitrate lyase and malate synthase was found in P3 which would indicate their association with a cell fraction lighter than the mitochondrion.

Sucrose density gradient centrifugation

The pellets P2 and P3 were combined and centrifuged on a sucrose density gradient. Fractions (1 ml) were collected and assayed for isocitrate lyase, malate synthase and cytochrome oxidase activities. The distribution of these enzymes and of protein content throughout the gradient is shown in Fig. 1. Cytochrome oxidase and protein show a peak at fractions 5 to 7 which is taken to correspond to the mitochondrial region. Malate synthase and

isocitrate lyase coincided in a peak of fractions 8 to 10 and this region, which is distinct from and of a lighter buoyant density than the mitochondrion, is regarded as corresponding to a separate particle, the glyoxysome. Malate dehydrogenase was also assayed in the gradient fractions and it showed a peak in the mitochondrial region (fractions 5 to 7) but the activity of the enzyme in all fractions was very low.

DISCUSSION

The growth conditions of the experimental material, the composition of the extraction medium and the severity of the homogenization can all affect the particulate enzyme titre in any localization study. In this work there was a definite separation of two glyoxylate-cycle enzymes from a mitochondrial marker enzyme in differential centrifugation and sucrose density gradient experiments. This indicates the presence of a glyoxysomal structure (Avers, 1971; Tolbert, 1971). For this to be of a lighter buoyant density than the mitochondrion is in contrast to *Neurospora crassa* (Kobr *et al.* 1969) but is in agreement with the situation in yeast reported by Szabo & Avers (1969). However, other workers have reported only soluble isocitrate lyase activity in extracts of certain fungi (Duntze *et al.* 1969; Cotter, La Clave, Wegener & Niederpruem, 1970; Perlman & Mahler, 1970) so that further clarification of the properties of the fungal glyoxysome is necessary (Avers, 1971).

Two isoenzymes of malate dehydrogenase were found in *Neurospora crassa* grown on acetate but only one of these occurred in cells grown on glucose (Zink & Shaw, 1968). Kobr *et al.* (1969) have shown that the latter is mitochondrial while the other is glyoxysomal. The apparent existence of three isoenzymes of malate dehydrogenase in *Coprinus* (unpublished observations), as in yeast (Atzpodien, Gancedo, Duntze & Holzer, 1968), complicates the interpretation of the fractionation data here. Whilst there was no evidence of malate dehydrogenase being associated with the postulated glyoxysome in *Coprinus*, the low activities of the preparations applied to the sucrose density gradients mean that a particulate location other than the mitochondrion cannot be ruled out.

The authors thank the Science Research Council for financial support, Dr L. A. Casselton for the strain of *Coprinus* and Miss L. Bullen for technical assistance.

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