THE ROLE OF ISOCITRATE LYASE IN *ASPERGILLUS NIDULANS*

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1. Introduction

Although the glyoxylate cycle has been demonstrated to occur in a number of moulds, including *Aspergillus niger* [1], *Neurospora crassa* [2–4] and *Rhizopus nigricans* [5], little is known of its genetic regulation in such eukaryotic organisms. It is the purpose of this paper to describe the isolation of mutants of *Aspergillus nidulans* devoid of isocitrate lyase activity. These mutants no longer grow on acetate, but revertants to growth on acetate have regained isocitrate lyase activity. Studies with wild-type cells show that this enzyme is inducibly formed prior to growth on acetate and is diluted out when acetate-grown cultures are transferred to media containing carbohydrates as carbon source. Taken together, these findings show that isocitrate lyase activity is necessary and sufficient for the growth of *A. nidulans* on acetate.

The availability of mutants devoid of isocitrate lyase activity has also permitted the location of the gene specifying this enzyme (*icl*), in linkage group *V*.

2. Experimental procedures

Media and routine techniques for genetic analysis in *Aspergillus nidulans* were those previously described [6, 7]. Cultures for the determination of isocitrate lyase activity were grown on defined liquid minimal medium [8] with the addition of 0.2 M potassium phosphate buffer, pH 6.5. Carbon sources were sterilised separately; sodium acetate and sodium glutamate having first been brought to pH 6.5. Cultures were started by the addition of suspensions of washed conidia (10^6 conidia/ml medium) and incubated at 37° with vigorous shaking on a gyrotary shaker.

Mycelium was harvested from 25 ml samples of culture on a millipore filter and washed with distilled water. Cell-free extracts for enzyme assay were made by sonicating the mycelium for 3 min in cold 40 mM phosphate buffer pH 7.0 and centrifuging at 12,000 *g*. Isocitrate lyase was measured with a SP 800 recording spectrophotometer. The method was that previously described [9], modified by omitting glutathione from the reaction mixture, using imidazole buffer at pH 7.0 and running the assays at 37°. The protein content of cell-free extracts was measured by the method of Lowry et al. [10], whereas this method was modified for the measurement of the total protein content of mycelial samples. For this purpose, washed mycelium from a 25 ml sample of the culture was sonicated for 3 min in cold distilled water, made to volume, and a sample of the total suspension extracted for 2 hr in the alkali reagent before addition of the Folin reagent. Cell debris was spun down before reading the extinction at 600 nm. Total protein was approximately one third of dry weight throughout the logarithmic phase of growth.

3. Results and discussion

3.1. *Isocitrate lyase activity in mycelium growing on different carbon sources*

The amounts of isocitrate lyase present in late log phase mycelium during growth on a number of carbon sources are shown in table 1. Low levels of enzyme were formed during growth on sucrose,

Table 1
Isocitrate lyase activity after growth on different carbon sources.

Carbon source	Concn. (M)	Time of harvesting (hr after inoculation)	Specific activity (μmoles glyoxylate formed/ mg protein/hr)
Acetate	0.1	24	25.8
Ethanol	0.1	41	11.3
Glucose	0.02	18	0.2
Sucrose	0.02	18	0.2
Glutamate	0.02	27	0.1
Glycerol	0.04	21	0.3
Acetate and glucose	0.1 / 0.02	21	24.0
Acetate and glycerol	0.1 / 0.04	21	27.0
Acetate and glutamate	0.1 / 0.02	24	31.8
Acetate and sucrose	0.1 / 0.02	21	9.6

glucose, glycerol or glutamate, but high levels were found during growth on acetate or ethanol; these high levels were not significantly reduced by the presence of glucose, glycerol or glutamate with acetate in the culture media. However, when sucrose was present with acetate the specific activity of isocitrate lyase was only about one half of that observed in the absence of sucrose. These findings are in agreement with the observation that *A. nidulans* utilises acetate preferentially from a mixture of acetate and sucrose [11].

3.2. *Transfer experiments*

The induction of isocitrate lyase was measured in an experiment in which mycelium growing in sucrose medium was transferred to medium containing acetate. The culture was divided into two and the mycelium harvested and washed aseptically at 37° by filtration. One half of the mycelium was transferred to acetate medium and the other returned to sucrose as a control. Incubation was continued and at intervals 25 ml samples were taken from each of the cultures to measure isocitrate lyase activity and total protein.

Fig. 1 shows the change in specific activity with time in the two cultures. In the mycelium returned to sucrose medium isocitrate lyase activity remained at a low level. However, isocitrate lyase was induced exponentially in the mycelium transferred to acetate from sucrose, finally reaching the maximum level of 31 units about 21 hr after transfer. In this culture there was a lag in growth of some 9 hr before any increase in total protein was detected, whereas no growth lag occurred in the mycelium returned to sucrose medium. Thus induction of isocitrate lyase precedes growth on acetate. In contrast, other experiments have shown that when acetate is added to a culture growing on glucose or sucrose there is no lag in growth, and isocitrate lyase formation begins immediately and at a high rate.

To examine the fate of isocitrate lyase upon removal of acetate, mycelium growing on acetate was transferred to a growth medium containing sucrose, or, as a control, returned to acetate. As shown in fig. 2, the culture returned to acetate continued to produce isocitrate lyase. However, in the culture transferred to sucrose isocitrate lyase synthesis did not continue; moreover, the curve for total enzyme per volume of culture remained almost horizontal for about three doublings (10 hr further incubation). It therefore appears that under the conditions of this experiment the enzyme is diluted out during growth on sucrose, and is not destroyed.

3.3. *Mutants devoid of isocitrate lyase activity*

Mutants unable to grow on acetate as sole carbon source were isolated by filtration enrichment following UV irradiation. Fifty five mutants were tested for isocitrate lyase activity. Six were found which showed no detectable enzyme activity after growth on a mixture of sucrose and acetate. These six mutants proved to be non-complementary to each other in heterokaryon tests, and are thus defective in the same function, the formation of isocitrate lyase.

The *icl* mutants have been located in linkage group *V* and map close to the *fac A* marker, with about 1% recombination. *fac A* Mutants, which are also unable to utilise acetate, were isolated [12] as

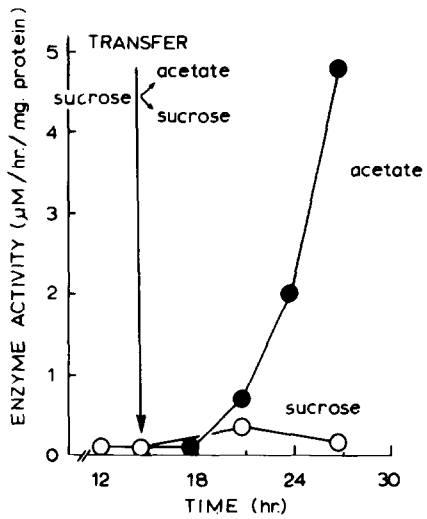

Fig. 1. Induction of isocitrate lyase upon transfer of mycelium from sucrose growth medium to acetate medium. A culture grown for 14½ hr in a sucrose medium was divided into two parts and harvested by filtration. The mycelium was then resuspended in either acetate or sucrose growth medium. 25 ml samples were taken from both cultures at the intervals shown during further incubation and isocitrate lyase activity measured.

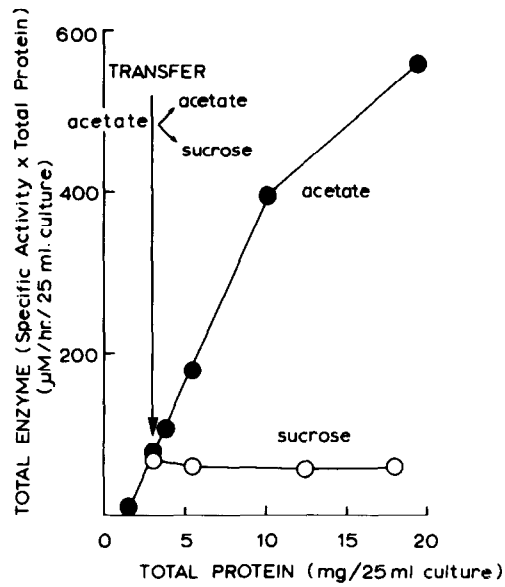

Fig. 2. Stability of isocitrate lyase upon transfer of mycelium from acetate growth medium to sucrose medium. A culture grown for 21 hr in acetate medium was divided into two parts and harvested by filtration. The mycelium was then resuspended in growth medium containing either sucrose or acetate. 25 ml samples were taken from both cultures at intervals during further incubation and isocitrate lyase activity and total protein measured. The curves show the change in total enzyme (specific activity X total protein) with increasing total protein.

mutants resistant to fluoroacetate in the presence of glucose; they have been shown [11] to lack acetyl CoA synthetase activity. All the *icl* mutants obtained complement with *fac A* in the heterokaryon.

3.4. *Reversion of icl mutants*

A number of revertants capable of growth on acetate were isolated from three of the *icl* mutants, and twenty of these revertants were tested for isocitrate lyase activity. All were found to have regained the ability to produce the enzyme when grown on acetate, although the specific activities observed ranged from 1% to 100% of wild-type. Those revertants with low enzyme activity typically grow poorly upon acetate.

These results therefore demonstrate that in *A nidulans*, as in *E. coli* [13], the glyoxylate cycle plays a necessary role in growth on acetate. Since the techniques for genetic analysis of *A. nidulans* are well established [6, 7], it is hoped to study the regulation of expression of the genes specifying this cycle in this eukaryotic organism, and to compare it with that operating in prokaryotic organisms such as *E. coli* [14].

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