Malic enzyme is a major source of NADPH for lipid accumulation by *Aspergillus nidulans*

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Malic enzyme was shown to be a major source of NADPH for the synthesis of storage lipid by *Aspergillus nidulans*. A previously produced mutant lacking malic enzyme activity (*acuK248*) accumulated only half the lipid (12%, w/w, of cell dry weight) accumulated by strains of *A. nidulans* possessing malic enzyme. When cultivated under conditions designed not to promote the production of storage lipid all three strains of *A. nidulans* studied contained equivalent amounts of lipid (5%, w/w, of cell dry weight). Thus malic enzyme did not appear to be limiting the synthesis of metabolically active lipid (e.g. membrane lipids). No evidence of a role for malic enzyme in the desaturation of fatty acids was found.

Keywords: lipid biosynthesis, malic enzyme, *Aspergillus nidulans*

INTRODUCTION

Malic enzyme [malate dehydrogenase (decarboxylating) (NADP⁺) (EC 1.1.1.40)] catalyses the reaction:

This enzyme has been detected in a range of fungi though its cellular function remains obscure. Some authors report that its main function is in the metabolism of pyruvate (McCullough & Roberts, 1974; Zink, 1972; Zink & Katz, 1973) whilst others have presented evidence that malic enzyme is also a major source of NADPH for *de novo* lipid biosynthesis and desaturation (Evans & Ratledge, 1985; Kendrick & Ratledge, 1992a).

In this communication the importance of malic enzyme with respect to lipid metabolism in *Aspergillus nidulans* has been investigated. This fungus was chosen because it is oleaginous, accumulating up to 25% of its dry weight as lipid (Sharma *et al.*, 1981), and mutants with altered malic enzyme activities are available (McCullough & Roberts, 1974).

METHODS

Chemicals. All fine chemicals were obtained from Sigma. Sodium salts were used throughout.

Maintenance of fungi. The three strains of *A. nidulans* used were auxotrophic with regard to *p*-aminobenzoic acid. Strain *pabaA1* was unaffected with respect to its malic enzyme activity and is described throughout as wild-type. The other two strains were mutants unable to utilize acetate, originally produced by McCullough and co-workers (McCullough & Roberts, 1974; Armitt *et al.*, 1976). One of these, *acuK248*,

was reported to lack malic enzyme activity, whilst the other, *acuF205*, has been reported to possess elevated malic enzyme activity (McCullough & Roberts, 1974). The mutants were routinely maintained on Pontecorvo medium agar plates (Pontecorvo *et al.*, 1953) and were stored at 4 °C.

Production of conidial suspensions. Conidia were harvested in 0.8% (w/v) Tween 80. Conidia were dislodged from the surface of agar plates by gentle rubbing with a sterile cotton bud. The harvested conidia from a number of plates were pooled and sedimented by centrifugation at 10000 *g* for 5 min at 4 °C. The conidial pellet was suspended in a small volume of 0.8% Tween 80 and the conidial concentration determined using a Thoma counting chamber.

Cultivation of fungal biomass. The fungus was grown in 1 litre Erlenmeyer flasks containing 200 ml liquid medium at 37 °C, with constant shaking at 145 r.p.m. The medium used was that of Das & Sen (1983) containing (g l⁻¹): glucose, 40; NaNO₃, 2; MgSO₄·7H₂O, 0.5; KCl, 0.5; ZnSO₄·7H₂O, 0.01; FeSO₄·7H₂O, 0.01; and *p*-aminobenzoic acid, 0.002. This medium was designated HAM ('high-nitrogen' *Aspergillus* medium). When high lipid yields were required the medium was modified by decreasing the amount of NaNO₃ to 1 g l⁻¹; this medium was designated LAM ('low-nitrogen' *Aspergillus* medium). Culture flasks were inoculated with either 0.5 × 10⁶ or 3 × 10⁶ conidia ml⁻¹.

Production of cell extracts. Mycelia were harvested by filtration and washed with distilled H₂O. Samples of the culture filtrate were retained for separate analysis, being stored at -21 °C if necessary. Biomass was suspended in 100 mM KH₂PO₄/KOH buffer (pH 7.5) containing 20% (w/v) glycerol, 1 mM benzamidine and 1 mM DTT and disrupted by passage twice through a French press at 35 MPa. The resulting homogenate was centrifuged at 15000 *g* for 10 min at 4 °C and the supernatant used to determine enzyme

Table 1. Enzyme activities in *A. nidulans* grown on 'high-nitrogen' medium (HAM) for 40 h

Activities are presented as mean values $\pm$ standard error of the mean ($n = 3$) for replicate assays of a single extract. The experiment was repeated three times using extracts prepared from separate cultures; typical data are presented. ND, Activity not detected.

Enzyme	Enzyme specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]		
	Wild-type	<i>acuF205</i>	<i>acuK248</i>
ME	0.011 $\pm$ 0.003	0.016 $\pm$ 0.040	ND
ACL	0.051 $\pm$ 0.002	0.050 $\pm$ 0.006	0.050 $\pm$ 0.003
6-PGDH	0.260 $\pm$ 0.030	0.296 $\pm$ 0.062	0.313 $\pm$ 0.040
G-6-PDH	1.073 $\pm$ 0.031	1.170 $\pm$ 0.115	1.590 $\pm$ 0.195
MDH	8.540 $\pm$ 0.193	9.709 $\pm$ 0.271	9.754 $\pm$ 0.676
NADP ⁺ :ICDH	0.071 $\pm$ 0.005	0.060 $\pm$ 0.002	0.090 $\pm$ 0.008
PC	0.031 $\pm$ 0.003	0.030 $\pm$ 0.003	0.006 $\pm$ 0.001
PDH	ND	ND	ND
PDC	0.005 $\pm$ 0.001	0.010 $\pm$ 0.002	0.001 $\pm$ 0.001

* ME, malic enzyme; ACL, ATP:citrate lyase; 6-PGDH, 6-phosphogluconate dehydrogenase; G-6-PDH, glucose-6-phosphate dehydrogenase; MDH, malate dehydrogenase; NADP⁺:ICDH, NADP⁺-dependent isocitrate dehydrogenase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PDC, pyruvate decarboxylase.

activities. Protein concentrations were determined according to Bradford (1976), with BSA as a standard.

Estimation of enzyme activities. All enzyme activities were determined using continuous spectrophotometric assays following the oxidation or reduction of NAD(P)(H) at 340 nm, within 2 h of extract preparation. All assays were carried out at 37 °C.

Enzyme assays were, with the exception of that for NADP⁺-dependent isocitrate dehydrogenase, carried out as described in the literature with only minor alterations: malic enzyme was assayed as described by Hsu & Lardy (1969) with malate at 25 mM; ATP:citrate lyase as described by Srere (1959) at pH 8.6; 6-phosphogluconate dehydrogenase as described by Potremoli & Grazi (1966); glucose-6-phosphate dehydrogenase as described by Langdon (1966); malate dehydrogenase as described by Ochoa (1955); pyruvate decarboxylase and pyruvate dehydrogenase as described by Reed & Willms (1966); pyruvate carboxylase as described by Seubert & Weicker (1969). The assays for ATP:citrate lyase, pyruvate carboxylase, pyruvate decarboxylase and pyruvate dehydrogenase were supplemented with 10 mM sodium azide, to inhibit NADH oxidase.

NADP⁺-dependent isocitrate lyase activity was determined using an assay system containing 82 mM Tris/HCl (pH 8.0), 3 mM MgCl₂, 0.24 mM NADP⁺, 5 mM isocitric acid.

Determination of culture dry weights. Ten millilitres of a shake-flask culture was filtered through pre-weighed filter circles (Whatman no. 1), washed with distilled H₂O, then dried at 100 °C for 48 h and reweighed.

Analysis of culture filtrates. Glucose concentrations were determined using a GOD Perid test kit (Boehringer Mannheim). Citrate concentrations were determined using the method of Williamson & Corkey (1969). Malate concentrations were determined using an assay containing: 87 mM KH₂PO₄/KOH buffer (pH 7.5), 3 mM MgCl₂, 0.5 mM NADP⁺, 0.1 unit malic enzyme (chicken liver). The assay was initiated by the addition of 100 μl culture filtrate and

incubated at 30 °C for 20 min. The A₃₄₀ was determined against a reagent blank.

Lipid analysis. Freeze-dried mycelia were extracted with 2:1 (v/v) chloroform/methanol (Folch *et al.*, 1957) and the lipid content of the biomass determined gravimetrically. Fatty acids were methylated using trimethylsulphonium hydroxide (Butte, 1983) and analysed by GC using a packed column (200 $\times$ 0.4 cm, 10%, w/w, diethylene glycol succinate and 1%, w/w, H₃PO₄ on Chromosorb W-AW 80/100 mesh; Alltech Associates). The column was run at 200 °C with the injector and detector at 230 °C. Fatty acid methyl esters were identified by comparison with authentic standards.

Whole-cell lipid was separated into neutral, sphingolipid plus glycolipid, and polar lipid fractions (Kendrick & Ratledge, 1992b).

RESULTS

Enzymological analysis

For the estimation of enzyme activities in *A. nidulans*, a 'high-nitrogen' medium (HAM) was inoculated with 0.5×10^6 conidia ml⁻¹ and incubated for 40 h. Malic enzyme was readily detected, albeit at relatively low activity, in both the wild-type [11 nmol min⁻¹ (mg protein)⁻¹] and *acuF205* [16 nmol min⁻¹ (mg protein)⁻¹], but was undetectable in extracts prepared from *A. nidulans acuK248* (see Table 1).

Other enzyme activities that were also measured (see Table 1) were: ATP:citrate lyase, which is involved in lipid accumulation; glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP⁺-dependent isocitrate dehydrogenase, which are NADPH generators; and pyruvate dehydrogenase, pyruvate carboxylase and pyruvate decarboxylase, which are involved in pyruvate metabolism.

Table 2. Analysis of growth and lipid accumulation by *A. nidulans*

Data are presented as mean values $\pm$ standard error ($n = 3$).

	Wild-type	<i>acuF205</i>	<i>acuK248</i>
Grown on 'high-nitrogen' medium (HAM) for 40 h			
Initial glucose (g l ⁻¹)	46.2 $\pm$ 0.2	46.2 $\pm$ 0.2	46.2 $\pm$ 0.2
Residual glucose (g l ⁻¹)	41.1 $\pm$ 1.0	35.1 $\pm$ 0.7	38.7 $\pm$ 0.9
Cell dry weight (g l ⁻¹)	1.6 $\pm$ 0.5	2.9 $\pm$ 0.4	1.8 $\pm$ 0.3
Cell lipid (% w/w, of dry weight)	4.5 $\pm$ 0.7	4.7 $\pm$ 1.2	3.8 $\pm$ 1.6
Malate in culture filtrate (mg l ⁻¹)	17.6 $\pm$ 0.8	30.2 $\pm$ 3.7	15.6 $\pm$ 0.9
Cell yield [g dry weight (g glucose used) ⁻¹]	0.31	0.26	0.24
Grown on 'low-nitrogen' medium (LAM) for 70 h			
Initial glucose (g l ⁻¹)	44.6 $\pm$ 0.1	44.6 $\pm$ 0.1	44.6 $\pm$ 0.1
Residual glucose (g l ⁻¹)	27.0 $\pm$ 0.2	30.4 $\pm$ 1.6	25.9 $\pm$ 0.0
Cell dry weight (g l ⁻¹)	3.8 $\pm$ 0.2	4.9 $\pm$ 0.3	5.1 $\pm$ 0.1
Cell lipid (% w/w, of dry weight)	26.5 $\pm$ 1.6	23.5 $\pm$ 2.7	12.4 $\pm$ 1.7
Malate in culture filtrate (mg l ⁻¹)	26.5 $\pm$ 1.6	72.7 $\pm$ 0.6	138.8 $\pm$ 3.7
Cell yield [g dry weight (g glucose used) ⁻¹]	0.22	0.34	0.28

Lipid analysis

Analysis of the cellular lipid when *A. nidulans* was grown on 'high-nitrogen' medium demonstrated very little difference between the wild-type and mutant strains (see Tables 2 and 3). The amount of lipid accumulated was low, approximately 5% (w/w) of dry weight, and the fatty acyl composition of the cell lipids was similar (see Table 3). The predominant fatty acids were 16:0, 18:0, 18:1 and 18:2 (all > 5%, w/w, of the total lipid). Notably, the 18:2 content of the cell lipid was very high in all cases, constituting approximately 60% (w/w) of the total cell fatty acids.

When the wild-type was cultivated from an inoculum of 3×10^6 conidia ml⁻¹ for 70 h on the 'low-nitrogen' medium (LAM), to encourage lipid accumulation, analysis demonstrated both quantitative and qualitative changes in the cell lipid. The total lipid had increased to 26% (w/w) of the cell dry weight and the fatty acyl composition of this lipid was different from that of lipid produced on 'high-nitrogen' medium. Although the predominant fatty acyl constituents were the same, there was a decrease in the content of 18:2 and a concomitant increase in 18:0 and 18:1 so that all three of these fatty acids were present as approximately 20–30% (w/w) of the total cell lipid (see Table 3).

When *A. nidulans* was grown on 'low-nitrogen' medium for 70 h, differences between the lipid accumulation in the three strains of *A. nidulans* became apparent. Whilst the wild-type and *acuF205* accumulated equivalent amounts of cell lipid (approximately 25%, w/w, of cell dry weight), *acuK248* accumulated only half this amount (12%, w/w, of cell dry weight; see Table 2). This difference did not appear to be the result of impaired growth or glucose metabolism since all three strains achieved similar culture dry weights and cell yield (see Table 2). Qualitatively, the fatty acyl compositions of

Table 3. Major fatty acyl constituents of cell lipid from *A. nidulans*

Data are presented as mean values from duplicate experiments. UI, unsaturation index = [% monoene + 2 (% diene) + 3 (% triene)]/100.

Major fatty acids	Relative % (w/w) of total fatty acyl groups in cell lipid		
	Wild-type	<i>acuF205</i>	<i>acuK248</i>
Grown on 'high-nitrogen' medium (HAM) for 40 h			
16:0	18.9	18.4	17.6
18:0	5.9	6.2	6.5
18:1	8.8	12.0	9.2
18:2	61.0	58.8	61.1
UI	1.4	1.4	1.4
Grown on 'low-nitrogen' medium (LAM) for 70 h			
16:0	22.7	22.3	19.9
18:0	22.3	21.4	23.7
18:1	22.9	31.2	19.0
18:2	29.4	22.6	34.4
UI	0.9	0.9	0.9

the lipid accumulated by the three strains of *A. nidulans* under these conditions were similar, although some variation in the relative contents of 18:1 and 18:2 was apparent (see Table 3).

When the fungi were cultivated on 'low-nitrogen' medium for 70 h, separation of the cell lipid into neutral lipid, glycolipid and polar lipid fractions demonstrated that the majority of the accumulated lipid was neutral lipid. Neutral lipid (predominantly triacylglycerols) constituted approximately 85% (w/w) of the lipid in the

wild-type and *acuF205* and 77% (w/w) of the lipid in *acuK248*. Generally, the fatty acyl compositions of the three lipid fractions were similar to each other and resembled that of the whole-cell lipid for the wild-type and *acuF205* strains. The polar lipid fraction from *acuK248*, however, possessed a higher percentage of 18:2 and concomitantly less 18:0 and 18:1 than the other lipid fractions and the whole-cell lipid sample.

Analysis of culture filtrates

When the fungi were grown on 'high-nitrogen' medium (lipid-non-accumulating conditions), the concentrations of malate in the culture filtrates from all three fungi were similar and low (see Table 2). Under lipid-accumulating conditions (when the fungi were grown on 'low-nitrogen' medium), however, both *acuF205* and *acuK248* excreted higher levels of malate which were significantly greater than that produced by the wild-type: twofold and fourfold greater, respectively. No citrate was detected in the culture filtrates from any of the fungi.

DISCUSSION

Generally the activities of malic enzyme detected throughout this work were low, which is in agreement with the work of McCullough & Roberts (1974), who reported low activity of malic enzyme when *A. nidulans* was cultivated on glucose.

The enzymological data demonstrated that, of the enzymes examined, *acuK248* appeared to be lacking only malic enzyme activity. This is in accord with the conclusion that *acuK248* carries a mutation in the malic enzyme structural gene (McCullough & Roberts, 1974). Although the malic enzyme activity detected in *acuF205* was higher than that in the wild-type the difference was small and not significant. It appeared therefore that the greatly elevated malic enzyme activity reported for this strain (McCullough & Roberts, 1974) may only be evident when malic enzyme activity is stimulated by transfer of biomass to acetate-containing medium.

The lower activities of pyruvate carboxylase and pyruvate decarboxylase in *acuK248* (which were only 20% of that in the wild-type strain) indicated that the absence of malic enzyme activity affected pyruvate metabolism in this strain. This would be in accord with the conclusion of McCullough & Roberts (1974) that malic enzyme plays a role in the metabolism of pyruvate in *A. nidulans*, but we would suggest that this is of only minor metabolic significance in glucose-grown cells.

It is possible that the pyruvate decarboxylase activity detected was due to the decarboxylase activity of a dissociated pyruvate dehydrogenase complex. As pyruvate dehydrogenase activity was not detectable this possibility was not readily precluded. It is thought probable, however, that the pyruvate decarboxylase activity was due to the presence of a discrete pyruvate decarboxylase, associated with the growth of *A. nidulans* as pellets in shake flasks, rather than as a homo-

geneous culture. The mycelia in the centre of these pellets were likely to experience O_2 -limited conditions and thus possess the enzymes associated with the anaerobic metabolism of glucose, including pyruvate decarboxylase.

Neither pyruvate carboxylase nor pyruvate decarboxylase (either as a discrete enzyme or as part of the pyruvate dehydrogenase complex) has previously been implicated as having an important role in lipid metabolism. Therefore the differences noted in this study, concerning storage lipid biosynthesis by strains of *A. nidulans*, are thought to be a result of the presence and absence of malic enzyme activity.

When *A. nidulans* was grown on 'high-nitrogen' medium for 40 h the lipid content of the biomass was low and essentially identical in all three strains regardless of their malic enzyme activity. It is suggested, therefore, that under these conditions the cell lipid produced was crucial to the metabolic functioning of the cell and that the production of this 'metabolically active' lipid was not limited by the activity of malic enzyme. The fatty acyl composition of this lipid was interesting in that it contained a very high proportion of 18:2 (approximately 60%, w/w, of the total lipid), suggesting that 18:2 was important for the metabolic activity of *A. nidulans*, as well as indicating that malic enzyme was not involved in fatty acid desaturation.

When the fungi were grown on 'low-nitrogen' medium to promote the accumulation of storage lipid, the possession of malic enzyme activity appeared to be a key factor to engender lipid accumulation. The strain of *A. nidulans* lacking malic enzyme activity (*acuK248*) accumulated only half as much lipid as the strains possessing malic enzyme (the wild-type and *acuF205*). The fact that *acuK248* did accumulate some storage lipid showed that although malic enzyme activity was required for maximal production of storage lipid, the lack of this enzyme could be overcome to some degree at least.

It has been suggested that the function of malic enzyme in lipid biosynthesis is to supply NADPH for fatty acid synthase and desaturases (Evans & Ratledge, 1985; Kendrick & Ratledge, 1992a). In *acuK248*, although malic enzyme was absent, other NADPH-generating enzymes – NADP⁺-dependent isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase – were all detected at high activities. Indeed, in all strains of *A. nidulans*, the activities of these enzymes were many times that of malic enzyme. Thus it is difficult to envisage a deficiency of NADPH for lipid biosynthesis based solely on the absence of malic enzyme and if there exists a single cellular pool of NADPH. The activity of malic enzyme could only limit storage lipid biosynthesis in regard to the supply of reducing power if there was a separate and discrete cellular pool of NADPH produced by malic enzyme and preferentially channelled into fatty acid synthesis probably by direct coupling to fatty acid synthase.

The possibility that the lack of malic enzyme in *acuK248* was limiting storage lipid formation by, in some way, interfering with the production of cytosolic acetyl-CoA from citrate, as described by Evans & Ratledge (1985), perhaps by the accumulated malate inhibiting ATP: citrate lyase activity, was discounted. Although malate did accumulate in the culture filtrate of *acuK248*, a similar accumulation was observed in *acuF205* (albeit to a lesser extent) even in the presence of malic enzyme activity and this did not adversely affect lipid accumulation. Furthermore, malate at the concentrations detected, in the culture filtrate samples, did not cause a substantial inhibition of ATP: citrate lyase activity in *A. nidulans* as determined *in vitro*.

Although *acuK248* accumulated less storage lipid than the strains possessing malic enzyme, the lack of malic enzyme activity in this strain did not adversely affect fatty acid desaturation. The unsaturation index (UI) for the lipid accumulated by all three strains was identical regardless of the presence or absence of malic enzyme activity. Thus there was no evidence of a role for malic enzyme in the desaturation of fatty acids as has been reported for the oleaginous fungus *Mucor circinelloides* (Kendrick & Ratledge, 1992a).

The data obtained in this study thus demonstrate that malic enzyme activity is important for the accumulation of storage lipid in *A. nidulans* and support the hypothesis that this enzyme is important in the generation of NADPH for lipid biosynthesis in oleaginous organisms (Evans & Ratledge, 1985). To the best of our knowledge, this is the first report to demonstrate a direct link between malic enzyme activity and the degree of lipid accumulation by an oleaginous fungus.

Malic enzyme did not appear to be important, however, for the synthesis of the lipid required for the metabolic activity of the cell. This is analogous to the requirement of ATP: citrate lyase by oleaginous yeasts for significant lipid yields (Botham & Ratledge, 1979) whilst non-oleaginous yeasts lacking this enzyme still generated the lipid required for metabolic purposes.

ACKNOWLEDGEMENTS

Grateful thanks are extended to Dr H. Sealy-Lewis (Department of Biological Sciences, University of Hull) for the kind donation of the strains of *A. nidulans* used during this study, also to Mrs M. Ewing for her expert technical assistance. This work was supported by a research grant (21.27.67) from the Biotechnology and Biological Sciences Research Council, UK.

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Received 1 July 1996; revised 28 August 1996; accepted 5 September 1996.