THE ROLE OF MALIC ENZYME IN *ASPERGILLUS NIDULANS*

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1. Introduction

Malic enzyme (L-malate: NADP oxido-reductase [decarboxylating] EC 1.1.1.40 [1]) has been found in a wide range of organisms. Among the bacteria both NAD and NADP linked malic enzymes are present while in eukaryotes the NADP specific enzyme is usually found. It has been suggested [2,3] that the decarboxylation of malate provides pyruvate for amino acid synthesis as well as for acetyl Co-enzyme A under conditions where C4 dicarboxylic acids are plentiful. This is supported by evidence that malic enzyme activity in *Escherichia coli* [4], *Streptococcus* [5] and *Pseudomonas* [6] is maximal during growth on malate or immediate precursors of C4 dicarboxylic acids. However results with eukaryotic micro-organisms are not wholly in accord with this interpretation and a number of alternative functions have been proposed [7–9].

We report here that the NADP linked malic enzyme in *Aspergillus nidulans* is required for growth on carbon sources which are precursors of tricarboxylic acid cycle intermediates. The enzyme is probably induced by a C4 dicarboxylic acid and single gene mutants lacking malic enzyme activity fail to utilise acetate or glutamate for growth. We are not aware of any previous reports of mutants lacking malic enzyme.

2. Methods

For enzyme studies *Aspergillus nidulans* strains R21 (*paba*A1, *y*A2) was cultured aerobically in defined liquid minimal medium in which KNO_3 is the nitrogen source, as described previously [10]. Mycelium for assay of malic enzyme was harvested by filtration and sonicated (MSE 150 W ultrasonic disintegrator) in cold 80 mM Tris–HCl buffer pH 7.5 containing $MgCl_2$ to 10 mM. After centrifugation at 4°C and 10^4 g for 15 min the supernatant was decanted and dithiothreitol added to 2 mM. Enzyme assays were performed within 60 min after sonication. Malic enzyme was assayed at 37°C in an SP 800 recording spectrophotometer by measuring the malate dependent increase in extinction at 340 nm due to NADP reduction [11]. The reaction mixture of 1 ml contained 80 μmoles Tris–HCl buffer pH 7.5, 10 μmoles $MgCl_2$, 10 μmoles L-malate, 2 μmoles dithiothreitol, 0.25 μmoles NADP and cell free extract. There was no activity with NAD. The reaction was dependent upon NADP and malate, and the formation of pyruvate was verified by making the 2,4-dinitrophenylhydrazone [12] which was identified by paper chromatography [13]. Pyruvate kinase (EC 2.7.1.40) was assayed at 37°C following the procedure of Kapoor and Tronsgaard [14]. Protein was assayed by the method of Lowry et al. [15] in an aliquot of the cell free extract taken before the addition of dithiothreitol.

3. Results

3.1. *Thiol requirement for activity of malic enzyme*

Malic enzyme activity was unstable in cell free extracts in the absence of a thiol reagent (fig. 1). Thus during 3 hr at 0°C enzyme activity decreased by 50% whereas extracts containing dithiothreitol retained full activity for at least 6 hr. Extracts held at 0°C

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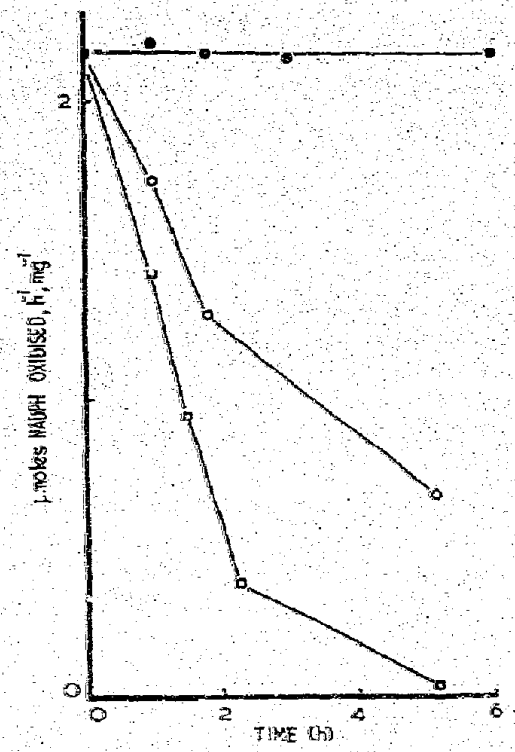

Fig. 1. The decay of malic enzyme activity in cell free extracts prepared from acetate grown R21 mycelium. Extracts were held at 0°C in the absence or presence of 2 mM dithiothreitol and assayed with or without dithiothreitol in the assay mixture. (●——●) stored with thiol, assayed with thiol present; (○——○) stored without thiol, assayed with thiol; (□——□) stored without thiol; assayed without thiol.

in the absence of dithiothreitol yielded a greater activity when the thiol reagent was included during assay, although the capacity for activation also decreased during storage. In the absence of dithiothreitol enzyme activity was completely inhibited by 5×10^{-5} M *p*-hydroxy-mercuribenzoate demonstrating that a sulphydryl group is involved in the reaction. An absolute thiol requirement has been reported for the maize leaf enzyme [16] and the involvement of a sulphydryl group at the active centre of the NAD linked malic enzyme in *Escherichia coli* has also been demonstrated [17]. Routinely dithiothreitol was added to cell free protein extracts immediately after sonication and was also included in the assay mixture.

3.2. *Malic enzyme activity during growth of R21 on different carbon sources*

Malic enzyme activity in mycelium growing on different carbon sources is shown in table 1. High activities were recorded during growth on acetate or glutamate, but low activities in mycelium grown on sucrose, glucose or glycerol. The high activity during growth on acetate was significantly decreased by the presence of sucrose in the growth medium but not by glucose, which is consistent with the observation [18] that *Aspergillus* utilises sucrose from a mixture of sucrose and acetate while glucose is largely excluded by acetate. Sucrose but not glucose also reduced activity during growth in the presence of glutamate. These results show that malic enzyme is either subject to carbon catabolite repression or is induced by a metabolite present at high concentration during growth on acetate and glutamate but not hexose or triose.

3.3. *Regulation of malic enzyme production*

The regulation of malic enzyme formation was investigated by testing the ability of acetate non-

Table 1
Malic enzyme activity during growth on different carbon sources

Carbon source	Concn. (M)	Time of incubation (hr)	Specific activity (μmoles NADPH oxidised $hr^{-1} \cdot mg^{-1}$)
Sucrose	0.02	18	0.28
Glucose	0.02	18	0.36
Glycerol	0.04	24	0.5
Acetate	0.1	24	2.3
Glutamate	0.02	24	3.6
Sucrose and Acetate	0.02 0.1	18	0.28
Glucose and Acetate	0.02 0.1	18	1.0
Sucrose and Glutamate	0.02 0.04	18	0.35
Glucose and Glutamate	0.02 0.04	18	1.5

Table 2
Malic enzyme activity in acetate non-utilising mutants

Strain	Lesion	Specific activity (μmoles NADPH oxidised, $hr^{-1} \cdot mg^{-1}$ protein)	Reference
*acu*A 204	Acetyl CoA synthase	0.1	Romano and Kornberg (1969)
*acu*D 202	isocitrate lyase	1.1	Armitt, Roberts and Kornberg (1970)
*acu*E 201	malate synthase	0.55	Armitt, Roberts and Kornberg (1971)
*acu*F 238	PEP carboxykinase	16.3	Armitt, McCullough and Roberts (in prep.)
*acu*G 223	FDPase	6.0	ditto
R21	none	2.0	

Malic enzyme activity in some *acu* mutants upon transfer of mycelium from sucrose and acetate grown medium to acetate medium. Cultures were grown for 18 h on a mixture of sucrose (2×10^{-2} M) and acetate (10^{-1} M), then harvested by filtration and the mycelium resuspended in fresh medium for 6 hr. Extracts were prepared and malic enzyme assayed as described in the text.

utilising (*acu*) mutants with known lesions [19] to develop enzyme activity under conditions which induce in the wild type. The mutants were first cultured on a mixture of sucrose and acetate which supports wild type growth rates, then transferred for 6 hr to fresh growth medium containing acetate which induced a high activity of malic enzyme in R21 (table 2). Malic enzyme activity was not formed in *fac*A which lacks acetyl co-enzyme A synthase [18] the initial step in acetate metabolism; however there was appreciable, though considerably less than wild type, induction in mutants lacking the glyoxylate cycle enzymes isocitrate lyase (*acu*D) [20] and malate synthase (*acu*E) [21]. More interesting however was the high activity of malic enzyme present in mutants lacking the gluconeogenic enzymes PEP carboxykinase (*acu*F) and fructose 1,6-diphosphatase (*acu*G). The very high activity of malic enzyme which develops in *acu*F mutants strongly suggests that the physiological inducer of this enzyme is a C4 acid or closely related metabolite. The alternative hypothesis, that malic enzyme is repressed by a metabolite from before FDP in glycolysis seems unlikely in view of the different responses of the two gluconeogenic mutants to acetate.

If as suggested [2,3] the function of this enzyme is to maintain the level of pyruvate during growth on precursors of TCA cycle intermediates then it might be expected that the activity of pyruvate kinase which normally provides pyruvate from glycolytic PEP would be minimal under these conditions. In fact pyruvate kinase activity during growth of R21 on acetate was 4 μmoles NADH oxidised $hr^{-1} \cdot mg^{-1}$ compared with values of 32 or 70 during growth on a mixture of sucrose and acetate or on sucrose respectively. There was no activation of pyruvate kinase by FDP such as occurs in *Saccharomyces* [22].

3.4. *Mutants lacking malic enzyme activity*

Among other groups of *acu* mutants [19] two failed to develop malic enzyme activity under a variety of growth conditions. Thus there was no

detectable enzyme activity in *acu*K or *acu*M mutants after growth with sucrose followed by transfer to acetate or glutamate or during growth on glycerol or a mixture of glycerol and glutamate. Both groups of *acu* mutants failed to grow with glutamate as sole source of carbon. The inability of these mutants to grow on acetate or glutamate, which also supported maximal malic enzyme activity in R21 strongly suggests that this enzyme is required for the utilisation of these compounds. Further work is in progress to characterise the lesions in *acu*K and *acu*M at least one of which is probably a structural gene for malic enzyme.

4. Discussion

Results presented here indicate that malic enzyme is required by *Aspergillus* for growth on carbon sources which are immediate precursors of TCA cycle intermediates. Since mutants which lack malic enzyme and have the capacity to form PEP carboxykinase [19] fail to grow on acetate or glutamate, it may be deduced that pyruvate kinase is not able to supply pyruvate from PEP under these conditions. The low level of pyruvate kinase activity found in extracts of acetate grown mycelium supports this interpretation. Amongs revertants of the malic enzyme mutants selected for growth on acetate we hope to find some altered in the control of pyruvate kinase formation.

Amongst other micro-organisms the unicellular alga *Euglena* [23] forms a novel malic enzyme in the presence of acetate as sole source of carbon. In the yeasts *Rhodotorula* has high malic enzyme activity during growth on acetate or malate but not glucose and the enzyme is absent from *Candida* and *Hansenula* [24]. The formation of the *Neurospora* enzymes is apparently induced during growth on sucrose and repressed by acetate while in *Fusarium* sucrose supports maximal activity with ammonium as nitrogen source but not nitrate. It is difficult to understand the behaviour of malic enzyme in these fungi if the function of the enzyme is only to provide pyruvate for biosynthesis.

Malic enzyme has also been implicated in energy metabolism and it has been suggested for mammalian cells [25,26] that a cytoplasmic transfer of hydrogen from NADH to NADP might occur by coupling the NAD linked malate dehydrogenase and malic enzyme. Differences in the behaviour of malic enzyme in different organisms might therefore be due to individual peculiarities of energy metabolism as well as the nature of the carbon source.

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