The Regulation of NADP-linked Isocitrate Dehydrogenase in *Aspergillus nidulans*

By JOAN M. KELLY† AND MICHAEL J. HYNES*

Department of Genetics, La Trobe University, Bundoora, Victoria 3083, Australia

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The regulation of NADP-linked isocitrate dehydrogenase (NADP-IDH) has been studied in wild-type and mutant strains of *Aspergillus nidulans*. In the wild-type strain studied, the levels of NADP-IDH vary in a similar way to those of acetamidase, acetyl-CoA synthase, isocitrate lyase and malate synthase under all growth conditions used. Similarly, *fac* mutants, which are altered in the regulation of these enzymes of acetate utilization, are affected in NADP-IDH levels in a parallel fashion, as are *cre* mutants, which show altered carbon catabolite repression of this group of enzymes. Possible functions of the NADP-IDH enzyme are considered.

INTRODUCTION

Metabolism of acetate via the glyoxylate cycle (Kornberg, 1966) by-passes the reaction catalysed by NADP-linked isocitrate dehydrogenase (EC 1.1.1.42; NADP-IDH), one of the steps of the tricarboxylic acid cycle. However, NADP-IDH activity is elevated in acetate-grown mycelia compared with sucrose-grown mycelia in the fungi *Aspergillus nidulans* (McCullough *et al.*, 1977) and *Neurospora crassa* (Kobr *et al.*, 1965; Flavell & Fincham, 1968). This was not observed in *Escherichia coli* (Holms & Bennett, 1971).

Studies on the regulation of acetamidase synthesis in *A. nidulans* have indicated that one control mechanism affecting this enzyme is also involved in the regulation of enzymes involved in acetate metabolism (Hynes, 1977). The acetamidase is inducible by acetate and other sources of acetyl-CoA, as are acetyl-CoA synthase (EC 6.2.1.1) and the glyoxylate cycle enzymes isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2). Mutants with lesions in any one of three genes, *facA*, *B* or *C*, (Apirion, 1965; Armitt *et al.*, 1976) are altered in induction of the acetamidase and the enzymes of acetate utilization (Armitt *et al.*, 1976; Hynes, 1977; Kelly & Hynes, unpublished). Mutations in *cre* genes lead to alterations in carbon catabolite repression of the acetamidase and the enzymes of acetate utilization (Hynes & Kelly, 1977; Kelly & Hynes, 1977 and unpublished). The striking parallels in the regulation of these enzymes in wild-type and mutant strains indicate that they are controlled together by one or more regulatory mechanisms. We show here that NADP-IDH is subject to similar controls to the acetamidase and acetate-utilization enzymes and is affected similarly in *fac* and *cre* mutants.

METHODS

Strains. The full genotypes of the strains used are given in Table 1.

Media. The media used were those of Cove (1966). Basic medium, designated –CN, lacked both a carbon and a nitrogen source.

† Present address: Department of Biology, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, U.K.

Table 1. *Full genotypes of strains used*

Strain	Genotype*	Reference
Wild type	<i>biA1; niiA4</i>	
<i>amd19</i>	<i>biA1; amd19; niiA4</i>	Hynes (1975)
<i>amd19; facA303</i>	<i>biA1; AcrA1; amd19; facA303; sB3; nicB8; riboB2</i>	Apirion (1965)
<i>amd19; facB101</i>	<i>wA3; amd19; facB101 riboB2 niiA4</i>	Apirion (1965)
<i>amd19; facC102</i>	<i>amd19; facC102</i>	Apirion (1965)
<i>cre-34</i>	<i>cre-34</i>	Kelly & Hynes (1977)
<i>creA204</i>	<i>biA1 creA204; niiA4</i>	Hynes & Kelly (1977)
<i>cre-34; creA204</i>	<i>creA204; cre-34; riboB2</i>	Kelly & Hynes (1977)
<i>creB15</i>	<i>biA1; creB15</i>	Hynes & Kelly (1977)
<i>cre-34 creB15</i>	<i>cre-34 creB15</i>	Kelly & Hynes (1977)
<i>creC27</i>	<i>biA1; creC27; niiA4</i>	Hynes & Kelly (1977)
<i>cre-34 creC27</i>	<i>biA1; cre-34 creC27; niiA4</i>	Kelly & Hynes (1976)
<i>acuD306</i>	<i>wA3; pyroA4; acuD306</i>	Armitt <i>et al.</i> (1976)
<i>acuE201</i>	<i>yA1 pabaA1 acuE201</i>	Armitt <i>et al.</i> (1976)

* For meanings of gene symbols, see Clutterbuck & Cove (1976).

Growth of strains. Strains were grown at 30 °C in a medium containing 1% (w/v) sucrose plus 20 mM-ammonium tartrate for 16 h, and aseptically transferred to the treatment media for a further 6 h before harvesting.

Preparation of cell-free extracts. Cell-free extracts were made immediately after harvesting by grinding 0.3 g mycelium with glass beads in 2.7 ml extraction buffer, and centrifuging to remove cellular debris.

Enzyme and protein determinations. NADP-IDH activity was determined by following the isocitrate-dependent reduction of NADP at 340 nm at 30 °C. The extract was made in 60 mM-potassium orthophosphate pH 6.5 plus 5 mM-MgCl₂ plus 1 mM-EDTA. The assay buffer contained 2.8 ml of the extraction buffer plus 0.1 mg NADP ml⁻¹ and 0.1 ml crude extract. The reaction was initiated by the addition of 0.1 ml of a 5.2 mg ml⁻¹ solution of sodium isocitrate. Enzyme determinations were done in duplicate and independent experiments indicated less than 20% variation in specific activities under a given set of growth conditions. Proteins were measured by the Lowry method. Units are expressed as nmol product formed min⁻¹ (mg protein)⁻¹.

RESULTS

Incubation of wild-type *A. nidulans* in media containing acetate, acetamide or L-threonine resulted in higher NADP-IDH levels compared to levels in mycelia grown in media containing glycerol, L-proline or sucrose as sole carbon sources, or in media lacking a carbon source (Table 2). Addition of acetate to sucrose-containing medium resulted in somewhat higher levels of NADP-IDH. These findings were consistent with induction by acetate or a metabolite of acetate, and closely resemble the pattern found for acetamidase, acetyl-CoA synthase and isocitrate lyase (Hynes, 1977), and malate synthase (Kelly & Hynes, unpublished). Strains lacking isocitrate lyase (*acuD306*) or malate synthase (*acuE201*) produced high NADP-IDH levels when grown in the presence of acetate (Table 2). This indicated that these enzymes are not required for inducer formation from acetate.

The *cre* mutants of *A. nidulans* are pleiotropically affected in carbon utilization (Hynes & Kelly, 1977; Kelly & Hynes, 1977). One of the effects of these mutations is to alter the response of acetamidase, acetyl-CoA synthase, isocitrate lyase and malate synthase to the repression caused by the presence of sucrose or glucose. The *creA204*, *creB15* and *creC27* mutations have been found to result in increased levels of these enzymes in the presence of sucrose. Similar effects were found for NADP-IDH with levels approximately twofold higher in the mutants compared with the wild type in acetate-grown mycelia in the presence, but not the absence, of sucrose (Table 3). The *cre-34* mutation has been shown to cause lowered levels of the acetamidase and acetate-utilization enzymes in the presence, but not in the absence, of sucrose and, furthermore, the *cre-34* mutation was found to be epistatic to

Table 2. *NADP-IDH activity under various growth conditions*

Strain*	Treatment medium† (additions to –CN medium)	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]
Wild type	10 mM-NH ₄ Cl	25
Wild type	1% (v/v) glycerol + 10 mM-NH ₄ Cl	32
Wild type	25 mM-L-proline	30
Wild type	1% (w/v) sucrose + 10 mM-NH ₄ Cl	38
Wild type	1% (w/v) sucrose + 25 mM-acetate + 10 mM-NH ₄ Cl	65
Wild type	25 mM-acetate + 10 mM-NH ₄ Cl	210
Wild type	1% (w/v) sucrose + 10 mM-NaNO ₃	40
Wild type	1% (w/v) sucrose + 25 mM-acetate + 10 mM-NaNO ₃	67
Wild type	25 mM-acetate + 10 mM-NaNO ₃	200
<i>amd19</i> ‡	25 mM-acetamide	150
<i>amd19</i>	25 mM-L-threonine	65
<i>acuD306</i>	25 mM-acetate + 10 mM-NH ₄ Cl	160
<i>acuE201</i>	25 mM-acetate + 10 mM-NH ₄ Cl	120

* Full genotypes appear in Table 1.

† Mycelium was grown for 16 h in 1% (w/v) sucrose + 10 mM-ammonium tartrate before transfer to the indicated treatment medium for a further 6 h.

‡ An *amd19* mutant strain was used to ensure high metabolism of acetamide to acetate (see Hynes, 1977).

Table 3. *Effects of cre mutations on NADP-IDH activities*

Strain*	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]				
	Treatment medium† (additions to –CN medium):				
	10 mM-NH ₄ Cl	1% sucrose + 10 mM-NH ₄ Cl	0.5% glycerol + 10 mM-NH ₄ Cl	25 mM-acetate + 10 mM-NH ₄ Cl	1% sucrose + 25 mM-acetate + 10 mM-NH ₄ Cl
Wild type	25	38	32	210	65
<i>cre-34</i>	23	37	29	220	40
<i>creA204</i>	28	28	29	200	120
<i>cre-34; creA204</i>	ND	ND	ND	250	52
<i>creB15</i>	30	51	31	230	140
<i>cre-34 creB15</i>	ND	ND	ND	250	63
<i>creC27</i>	32	46	29	260	120
<i>cre-34 creC27</i>	ND	ND	ND	240	44

ND, Not determined.

* Full genotypes appear in Table 1.

† Growth conditions as for Table 2.

creA204, *creB15* and *creC27* mutations for their effects on these enzymes (Kelly & Hynes, 1977). NADP-IDH levels in the *cre-34* mutant grown in sucrose plus acetate medium were slightly lower than those in the wild type, but were similar to the wild-type levels when the strains were grown in medium containing acetate alone. In double mutants it was found that the *cre-34* mutation was epistatic to the effects of the *creA204*, *creB15* and *creC27* mutations on NADP-IDH levels in the presence of sucrose plus acetate (Table 3). Therefore the *cre* mutations had effects on NADP-IDH parallel to those on the acetamidase and acetate-utilization enzymes.

Mutations in three genes, *facA*, *facB* and *facC*, have been shown to lead to fluoroacetate resistance, to decreased acetate utilization, and to low acetyl-CoA synthase activities in *A. nidulans* (Apirion, 1965; Armitt *et al.*, 1976). These mutations affect induction of acetamidase, isocitrate lyase and malate synthase in a characteristic and parallel fashion (Hynes, 1977; Kelly & Hynes, 1977 and unpublished). Whereas *facB* mutations greatly reduce induction by both acetate and acetamide, *facA* and *facC* mutations do not completely abolish acetate induction while greatly affecting induction by acetamide. NADP-IDH

Table 4. *Effects of fac mutations on NADP-IDH activities*

Strain*	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]		
	Treatment medium† (additions to –CN medium):		
	10 mM-NH ₄ Cl	25 mM-acetate + 10 mM-NH ₄ Cl	25 mM-acetamide
<i>amd19</i>	30	200	150
<i>amd19; facA303</i>	35	130	40
<i>amd19; facB101</i>	29	40	31
<i>amd19; facC102</i>	28	75	22

* Full genotypes appear in Table 1. Strains containing the *amd19* mutation were used to increase potential inducer formation by acetamidase activity (see Hynes, 1977).

† Growth conditions as for Table 2.

induction was investigated in strains containing *fac* mutations (Table 4). All the strains also contained the *amd19* mutation in order to increase potential inducer formation from acetamide by acetamidase activity (Hynes, 1977). All three *fac* mutations eliminated the induction of NADP-IDH in mycelia transferred to acetamide-containing medium. In mycelia transferred to acetate-containing medium, induction of the NADP-IDH was only marginally reduced in the *facA* mutant, still apparent in the *facC* mutant, but almost completely abolished in the *facB* mutant. Therefore the effects of *fac* mutations on NADP-IDH induction were similar to their effects on acetamidase and acetate utilization enzymes.

A possible function of NADP-IDH activity during growth in acetate media could be to act as a source of NADPH (Flavell & Fincham, 1968; McCullough *et al.*, 1977). The major pathway of NADP reduction in *A. nidulans* during growth on hexoses is the pentose phosphate pathway (Hankinson, 1974; Hankinson & Cove, 1974). The presence of nitrate in glucose-grown mycelia has been found to increase the levels of the enzymes of the pentose phosphate pathway, presumably to meet an increased demand for NADPH for nitrate reduction (Hankinson, 1974). NADP-IDH activity, however, was not affected by the presence of nitrate in glucose-grown mycelium (Hankinson, 1974). When acetate is the sole carbon source it is unlikely that the pentose phosphate pathway is the major source of NADPH, and NADP-IDH and NADP-linked malic enzyme (EC 1.1.1.40; NADP-ME) are possibly important as sources of NADPH. Therefore the effects of nitrate on NADP-IDH levels in acetate-grown mycelia was determined to see if nitrate stimulated activity. As shown in Table 2, no effects of nitrate on NADP-IDH were observed, which indicated either that NADP-IDH is not an important source of NADPH or that the levels of NADP-IDH may be sufficient in acetate grown conditions for generation of NADPH for nitrate reduction.

DISCUSSION

The results presented here indicate that NADP-IDH is regulated in a similar way to acetamidase and enzymes of acetate utilization. The *cre* mutations which affect carbon metabolite repression of these enzymes also affect NADP-IDH to a similar degree. Mutations in the *fac* genes also alter NADP-IDH regulation in a parallel fashion to this group of enzymes. That the effects of these mutations on the regulation of NADP-IDH is not due to secondary effects arising from alterations in the levels of isocitrate lyase or malate synthase is shown by the lack of effect on acetate induction of NADP-IDH in mutants lacking either of these enzyme activities. Additional studies on the effects of *fac* mutations on enzyme induction have led us to suggest that the *facB* gene is an important regulatory gene involved in induction of enzymes of acetate utilization, including NADP-IDH, while the *facA* and *facC* genes affect structural and enzymic components involved in acetyl-CoA synthase, isocitrate lyase and malate synthase activities and in the intracellular distribution of acetate metabolites (Kelly & Hynes, unpublished).

The high levels of NADP-IDH present in acetate-grown mycelia raise the question of the function of this enzyme under these growth conditions. Preliminary results indicate no differences in NADP-IDH isoenzyme pattern in sucrose-grown versus acetate-grown mycelia (W. McCullough, personal communication), and therefore it is likely to be the same gene product present at higher levels during growth on acetate, although it is possible that the intracellular distribution of the enzyme is different. If there is only one isocitrate dehydrogenase enzyme in *A. nidulans*, then one essential function of this enzyme would be to supply 2-oxoglutarate for biosynthetic pathways, and therefore this enzyme would be required during growth on either sucrose or acetate in the absence of an alternative source of 2-oxoglutarate. Another function for NADP-IDH may be to supply NADPH. The pentose phosphate pathway is probably mainly responsible for NADPH production during growth on hexoses but not during growth on acetate, where the activities of NADP-IDH and NADP-ME may be the main sources of NADPH. This may explain why the levels of NADP-IDH are increased during growth on acetate. We have also observed that NADP-ME activity is somewhat increased during growth on acetate (Kelly & Hynes, 1981). In addition, during growth on L-proline as the sole carbon source, NADP-IDH levels are not affected (Table 2) while NADP-ME levels are very high (Kelly & Hynes, 1981). This suggests that during growth on acetate both NADP-ME and NADP-IDH generate NADPH, while during growth on L-proline only NADP-ME is responsible for NADPH generation.

No mutants lacking NADP-IDH are known in *A. nidulans*. If the sole function of this enzyme is to generate NADPH during growth on acetate, then mutants lacking this enzyme might show decreased growth on acetate as the sole carbon source. No mutants lacking NADP-IDH were found amongst mutants selected for inability to utilize acetate (Armitt *et al.*, 1976; McCullough *et al.*, 1977), although it is possible that such mutants might have been too leaky to have been selected. If the functions of NADP-IDH are both to provide 2-oxoglutarate and to generate NADPH during growth on acetate, then mutants lacking this activity might require supplementation by sources of 2-oxoglutarate such as L-proline and L-glutamate and show at least slightly decreased growth on acetate as the sole carbon source.

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