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Induction of the Acetamidase of *Aspergillus nidulans* by Acetate Metabolism

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Growth tests and enzyme determinations strongly suggest that the acetamidase of *Aspergillus nidulans* is induced by a product of acetate metabolism rather than the substrate, acetamide. The *cis*-dominant mutation, *amdI*9, which is closely linked to *amdS*, the structural gene for the acetamidase, results in greatly increased sensitivity to induction by acetate metabolism. Propionate, L-threonine, and ethanol also result in acetamidase induction. Mutations in the *facA*, *facB*, and *facC* genes, which lead to low levels of acetyl-coenzyme A synthase, are epistatic to the *amdI*9 mutation for strong growth on acetamide medium and abolish acetamide and propionamide induction of the acetamidase and isocitrate lyase enzymes. Acetate, L-threonine, and ethanol, however, can induce these enzymes in strains containing *facA* and *facC* lesions but not in strains containing a *facB* lesion. The evidence suggests that acetamidase and isocitrate lyase may be induced by a similar mechanism.

Aspergillus nidulans can use acetamide as a source of carbon and nitrogen and produces an acetamidase enzyme that is induced during growth in the presence of acetamide (7). Strains carrying the *cis*-dominant mutation, *amdI*9, closely linked to *amdS*, the structural gene for the acetamidase, have very high levels of acetamidase when grown in acetamide-containing media (9). Preliminary investigations of *amdI*9 strains showed that a metabolite of acetate rather than acetamide itself might be the true inducer. Evidence is presented here showing that growth on medium containing acetate or other sources of acetyl-coenzyme A (Ac-CoA) induces the acetamidase.

Apirion (1) isolated *facA*, *facB*, and *facC* mutants by selecting for resistance to fluoroacetate. Mutations in each of these genes lead to poor growth on acetate as the sole source of carbon, and *facA* and *facC* mutants have also been isolated by direct selection for acetate nonusers (2). Levels of Ac-CoA synthase and regulation of glyoxalate bypass enzymes are affected by mutations in each *fac* gene (2). Therefore, the effects of *fac* mutations on induction of the acetamidase were investigated. The results indicate that the pattern of induction of the acetamidase and isocitrate lyase enzymes are very similar and that the *fac* genes have a complex role in induction.

MATERIALS AND METHODS

Strains. Details of strains used are described in Table 1.

Media and growth conditions. The standard minimal medium used was that of Cove (6), where 1% (wt/vol) glucose is the sole carbon source and no nitrogen source is present. Nitrogen sources and auxotrophic supplements were added to the medium. In many cases, glucose was omitted from this medium (designated −CN medium) and carbon and nitrogen sources were added. The acetate medium used was that of Apirion (1). Ammonium (NH_4^+) was added as ammonium tartrate. Growth tests were performed with solid medium (1% Difco agar) and incubating at 37°C for 2 to 3 days. Mycelium was grown at 30°C in a Gallenkamp orbital incubator and harvested as described previously (8).

Preparation of crude extracts. Extracts of harvested mycelium were made as described (8). For acetamidase assays, extracts were made in 100 mM sodium orthophosphate buffer, pH 7.2. Where isocitrate lyase and Ac-CoA synthase activities were to be assayed, extracts were made in 100 mM potassium orthophosphate buffer, pH 7.5.

Enzyme assays. Acetamidase (EC 3.5.1.4) was assayed by the method described before. Ac-CoA synthase (EC 6.2.1.1) and isocitrate lyase (EC 4.1.3.1) were assayed by the methods described by Armitt et al. (2). All enzyme specific activities are expressed as milliunits per milligram of protein, where one unit is the amount of enzyme that catalyzes the production of 1 μmol of product per min.

RESULTS

Growth tests and acetamidase determinations on wild-type and *amdI*9 strains. It has been previously noted that wild-type *A. nidulans* can use acetamide as a sole nitrogen source in the presence of 1% glucose much bet-

TABLE 1. *Details of strains used in this work*

Strain[a]	Genotype[b]	Origin or reference
Wild type	*biA1 niiA4*	15
amdI9	*biA1 amdI9 niiA4*	9
amdI9 facA303 ...	*biA 1 acrA1 facA303 sB3 nicB8 riboB2*	Isolate from cross
amdI9 facC102 ...	*amdI9 facC102*	Isolate from cross
amdI9 facB101 ...	*wA3 amdI9 niiA4 facB101 riboB2*	Isolate from cross
facA303	*biA1 galA1 pyroA4 facA303 sB3 nicB8 niiA4*	Isolate from cross
facC102	*wA3 pyroA4 facC102*	Isolate from cross
facB101	*wA3 facB101 riboB2*	Isolate from cross
acuD306	*wA3 pyroA4 acuD306*	2
amdI9 facB501 ...	*biA1 amdI9 niiA4 facB501*	This paper
amdI9 facB502 ...	*biA1 amdI9 niiA4 facB502*	This paper
amdI9 facB507 ...	*biA1 amdI9 niiA4 facB507*	This paper

[a] Strains containing *facC*, *facB*, and *acuD* lesions were kindly provided by C. F. Roberts.
[b] For meaning of gene symbols other than those described here see Clutterbuck (5).

ter than in the presence of 1% sucrose (8). Romano and Kornberg (16) have shown that during growth of *A. nidulans* in medium containing both acetate and glucose, glucose is partially excluded and acetate is metabolized via the glyoxalate cycle. In contrast, sucrose is preferentially used from a mixture of sucrose and acetate (3, 16). The differential effect of glucose and sucrose on acetamide utilization can be explained, in the light of these results, by proposing that metabolism of acetate produced from acetamide by the acetamidase results in induction of the enzyme. Repression of acetate metabolism by sucrose leads to very low levels of induction, whereas repression by glucose is much weaker and some induction occurs in its presence.

Acrylamide is a substrate for the acetamidase but does not result in induction (12). Therefore, strains producing high uninduced enzyme levels can grow strongly, whereas wild-type strains grow poorly when acrylamide is the sole nitrogen source in the presence of sucrose or glucose. However, when acetate is present as the sole carbon source, wild-type strains show considerable growth on acrylamide as the sole nitrogen source, suggesting that acetate can result in induction of the acetamidase.

Growth of *amdI9* mutants on acetamide as either the sole nitrogen source in the presence of other carbon sources or as the sole carbon and nitrogen source is stronger than wild type. Use of acrylamide as the sole nitrogen source in the presence of acetate as the carbon source is greater for *amdI9* mutants than for wild-type strains. Furthermore, though wild-type strains grow very poorly on medium containing both glucose and acetate or sucrose and acetate with acrylamide as the sole nitrogen source, *amdI9* mutants grow quite strongly. These observations suggest that the *amdI9* mutation results in increased sensitivity to induction during acetate metabolism. Even the low level of acetate metabolism in the presence of sucrose is sufficient to give substantial induction in *amdI9*-containing strains. Further support for this suggestion is that the presence of ethanol (0.5%) allows acrylamide to be used as a nitrogen source in the presence of sucrose by *amdI9* strains. It is known that ethanol is metabolized via Ac-CoA in *A. nidulans* (M. M. Page and D. J. Cove, Biochem. J. 127:17p, 1972).

Preliminary assays showed that addition of 10 mM acetate or 10 mM acetamide to glucose-grown cultures of either wild-type or *amdI9* strains induced the acetamidase to a similar extent. Furthermore, addition of 10 mM acetamide to cultures growing in 1.2% acetate medium did not result in additional acetamidase induction.

Further studies on acetamidase induction were done on cultures grown in glucose plus ammonium medium for 16 h and then transferred to medium lacking either a carbon or nitrogen source (−CN medium) to which was added various potential sources of induction (Table 2). Acetamide, acetate, propionamide, and propionate induced two- to fourfold in the wild-type strain and about 10-fold in the *amdI9* strain. Butyramide, a relatively weak substrate for the acetamidase (10), did not lead to induction, whereas butyrate weakly induced the *amdI9* strain. Ethanol gave some induction in the wild-type strain and considerable induction in the *amdI9* mutant. L-Threonine is probably metabolized via Ac-CoA in fungi (17). *A. nidulans* mutants lacking the glyoxalate bypass enzymes are unable to utilize L-threonine as a sole carbon source (unpublished observations). L-Threonine was therefore tested as a source of induction and was found to induce the wild-type strain about threefold and the *amdI9* mutant about 10-fold. Induction by threonine was not additive with that by acetate.

TABLE 2. *Effect of potential inducers on acetamidase activities in wild-type and amdI9 strains*

Potential inducer added to −CN medium[a] (mM)	Acetamidase sp act	
	Wild type	*amdI9*
None	32	44
Acetamide (5)	84	400
Acetamide (10)	121	478
Acetate (10)	89	372
Propionamide (10)	70	368
Propionate (10)	111	513
Butyramide (5)	25	50
Butyrate (5)	ND[b]	126
Acrylate (5)	46	175
Ethanol (0.5%)	54	373
L-Threonine (5)	91	409
L-Threonine (5) + acetate (5)	ND	440
L-Alanine (5)	ND	51
Glycine (5)	ND	62
Citrate (5)	ND	40
Isocitrate (5)	ND	59
Malate (5)	ND	45
Succinate (5)	ND	43
Pyruvate (5)	ND	78

[a] Mycelium was grown for 16 h in 1% glucose + 20 mM NH_4^+ medium and then transferred to medium lacking a carbon or nitrogen source (−CN medium) to which was added potential sources of induction for 4 h before harvesting. All acids were added as the sodium salt with the pH adjusted to 6.5.

[b] ND, Not determined.

Acrylate, the product of acrylamide hydrolysis, did not cause increased enzyme levels in the wild type, but induced the *amdI9* strain about fourfold. This suggests that acrylate may be metabolized only to a limited extent. Furthermore, acrylamide is not a significant sole carbon source for *A. nidulans*. Therefore, the absence of induction by acrylamide in the presence of glucose, which is likely to repress acrylate metabolism, is not surprising.

Various compounds of the tricarboxylic acid cycle did not result in significant induction in the *amdI9* strain, but pyruvate resulted in weak induction. Pyruvate is a very poor sole carbon source for *A. nidulans*.

Effect of acetate utilization mutations on amide utilization and acetamidase induction. If acetate metabolism is required for acetamidase induction, then it would be expected that mutations affecting acetate utilization would affect growth on acetamide as a nitrogen source. Strains containing *facA*, *facB*, or *facC* lesions grow weakly compared with wild-type strains on glucose-acetamide or sucrose-acetamide media. In contrast, strains lacking isocitrate lyase or malate synthase due to lesions in the *acuD* and *acuE* genes, respectively (2), are unaffected in growth on acetamide as the sole nitrogen source.

Double-mutant strains containing *amdI9* and acetate utilization lesions were isolated from appropriate crosses, and their genotypes were confirmed by outcrossing to wild-type strains. The *amdI9 fac* double mutants grew poorly on acetamide media, showing that *fac* lesions are completely epistatic to *amdI9*. The *acuD306* mutation, however, was not epistatic to *amdI9*.

New *facB* mutants were isolated by selecting for spontaneous fluoroacetamide-resistant derivatives of the *amdI9* strain. Fluoroacetamide, when present in acetate medium, is very toxic to strains that produce high levels of the acetamidase because the acetamidase converts the fluoroacetamide to fluoroacetate (13). Four mutants that could grow only weakly on acetate medium were found among fluoroacetamide-resistant mutants of the *amdI9* strain. Each of these had a *facB* mutation since in heterozygous diploids they did not complement the *facB101* mutation for growth on acetate but did complement *facA* and *facC* mutations. Leaky growth on acetate is a characteristic of *facB* mutants (2). The mutations were designated *facB500*, 501, 502, and 507. Each of these new *facB* mutations was found to be epistatic to the *amdI9* mutation for growth on acetamide as the sole nitrogen source in the presence of glucose or sucrose.

As predicted by the growth tests, *facA*, *facB*, and *facC* mutations eliminated induction by acetamide and also propionamide (Table 3). Surprisingly, however, the *facA* and *facC* mutations did not greatly affect induction by acetate and only had slight effects on induction by propionate. L-Threonine, which is probably catabolized directly to Ac-CoA, gave significant induction in strains with either a *facA* or *facC* lesion. Ethanol induction of *amdI9* strains was affected by the presence of the *facA* and *facC* lesions under 4-h growth conditions in the presence of inducer, but, as shown in Table 4, with 6-h growth ethanol did give considerable induction in these strains. The *facB* mutants had low enzyme activities, and all showed little response to any sources of induction, with acetate and propionate giving less than twofold induction. Strains containing *facB502* and *facB507* gave results similar to the *amdI9 facB501* strain.

Effect of *fac* lesions on induction of acetamidase, Ac-CoA synthase, and isocitrate lyase. Since *fac* lesions have been shown to affect the enzyme Ac-CoA synthase and the induction of isocitrate lyase (2, 16), the levels of these enzymes in response to different sources of induction were determined (Table 4). These induction conditions were altered from the ones

TABLE 3. *Effect of fac mutations on induction of the acetamidase by various sources of induction*

Strain	Acetamidase sp act with:[a]						Ethanol
	None	Acetamide	Acetate	Propionamide	Propionate	L-Threonine	
Wild type	32	84	89	70	111	91	54
*facA*303	55	48	121	45	83	110	ND[b]
*facB*101	39	37	69	36	35	36	ND
*facC*102	29	36	98	25	56	48	ND
*amdI*9	44	478	372	368	513	409	373
*amdI*9 *facA*303	53	50	445	75	246	221	59
*amdI*9 *facC*102	41	45	353	52	251	179	75
*amdI*9 *facB*501	19	20	34	22	41	44	15
*amdI*9 *facB*101	16	26	28	ND	ND	28	ND

[a] Source of induction added to −CN medium. Mycelium was grown for 16 h in 1% glucose + 20 mM NH_4^+ medium and then transferred to −CN medium containing potential inducers for 4 h before harvesting. Inducers were present at 10 mM except for L-threonine (5 mM) and ethanol (0.5%).

[b] ND, Not determined.

TABLE 4. *Effect of fac mutations on the induction of acetamidase, Ac-CoA synthase, and isocitrate lyase in amdI9-containing strains*

Strain	Inducer[a]	Enzyme sp act		
		Acetamidase	Ac-CoA synthase	Isocitrate lyase
*amdI*9	None	29	10	1
	Acetamide	330	57	115
	Acetate	421	119	165
	Ethanol	381	93	82
	L-Threonine	316	18	38
*amdI*9 *facA*303	None	29	9	2
	Acetamide	26	0	2
	Acetate	405	5	72
	Ethanol	105	8	60
	L-Threonine	249	2	16
*amdI*9 *facB*101	None	8	7	2
	Acetamide	18	1	1
	Acetate	45	4	5
	Ethanol	15	17	9
	L-Threonine	29	21	12
*amdI*9 *facC*102	None	28	11	2
	Acetamide	52	11	7
	Acetate	387	20	68
	Ethanol	327	51	121
	L-Threonine	201	27	30

[a] Mycelium was grown for 16 h in 1% sucrose + 20 mM NH_4^+ and then transferred to −CN medium containing the inducer and 20 mM NH_4^+ for 6 h before harvesting. Inducers were added at 5 mM except for ethanol (0.5%).

described above by extending the time of incubation to 6 h and including ammonium in the medium, since these conditions maximize the levels of the enzymes of acetate metabolism (J. M. Kelly and M. J. Hynes, unpublished data). Ammonium does not repress the acetamidase under conditions of carbon limitation (7).

As expected, the *fac* lesions resulted in low Ac-CoA synthase activities under all conditions, with *facC*102 having the least effect. In the *fac*$^+$ strain acetamide, acetate, and ethanol all resulted in substantial induction of the Ac-CoA synthase, whereas the effect of L-threonine was much less.

All sources of acetamidase induction also induced the isocitrate lyase enzyme in the *fac*$^+$ strain. In *facA* and *facC* strains, induction of both isocitrate lyase and acetamidase by acetamide was greatly reduced, but induction by acetate, ethanol, and L-threonine was only slightly affected. The presence of the *facB* lesion greatly reduced induction of both enzymes

by all compounds. Thus, induction of the acetamidase had a parallel induction pattern to isocitrate lyase in the *fac* mutants.

DISCUSSION

The results described here show that some product of acetate metabolism is involved in acetamidase induction, and that acetamide probably induces the enzyme by virtue of its conversion to acetate. This leads to the prediction that acetamide will not induce the synthesis of inactive enzyme protein in nonleaky structural gene mutants since no inducer could be formed from acetamide.

Some evidence indicates that the inducer is Ac-CoA or a close derivative. L-Threonine is a strong inducer and is probably metabolized directly to Ac-CoA (17), and the *acuD*306 mutation, which leads to loss of isocitrate lyase (2, 3), does not affect the growth of *amdI*9 strains on acetamide media. In addition, various tricarboxylic acids or sources of tricarboxylic acids do not result in induction. However, the complexity of the effects of *fac* mutations on induction of the acetamidase by various sources of Ac-CoA indicates that the situation may not be this simple. The *facA* gene has been thought to be the structural gene for Ac-CoA synthase (2, 16), and preliminary studies on a temperature-sensitive *facA* allele indicate increased thermolability of the enzyme in crude extracts (Kelly and Hynes, unpublished data). Therefore, it might have been expected that *facA* mutations would completely block induction by acetate, propionate, and ethanol. However, the data presented here show that acetate and ethanol induce both acetamidase and isocitrate lyase in the *amdI*9 *facA*303 strain, whereas induction by acetamide is completely abolished. Similar results have been found for the *amdI*9 *facC*102 strain, which is less stringently affected in Ac-CoA synthase activity than the *facA*303 strain. Therefore, the effects of *facA* and *facC* lesions on induction depend on the source of the inducer.

In contrast to the effects of *facA* and *facC* lesions on induction, *facB* lesions strongly affect induction of both the acetamidase and isocitrate lyase enzyme by all sources of Ac-CoA. This may indicate that the *facB* gene product has a direct regulatory role in induction, while the *facA* and *facC* genes may influence the intracellular distribution of inducer. Clearly the role of the three *fac* genes in Ac-CoA metabolism requires further study. Some of the puzzling aspects of these genes have been previously discussed by Armitt et al. (2).

A significant feature of the effects of *fac* mutations on induction is that the acetamidase and isocitrate lyase enzymes are affected in a very similar fashion. This may indicate that the same regulatory mechanism is involved in the control of both enzymes. It will be of interest to investigate the effects of *fac* lesions on induction of malate synthase, the second enzyme of the glyoxalate bypass.

Since acetate metabolism is repressed during growth in the presence of sucrose (and to a lesser extent glucose) medium, induction of the acetamidase by acetamide and acetate is greatly restricted in the presence of sucrose. The *amdI*9 lesion apparently causes increased sensitivity to induction so that strains containing this mutation can grow much more strongly on acetamide as the sole nitrogen source in the presence of sucrose than wild-type strains due to induction by the very low levels of acetate-derived inducer present.

Strong support for this hypothesis has been found in studies on *cre* mutations. Strains containing these mutations have derepressed levels of both Ac-CoA synthase and isocitrate lyase enzymes in the presence of sucrose or glucose and grow more strongly than wild-type strains on sucrose-acetamide medium (11; unpublished data). Furthermore, another mutation leads to super-sensitivity to glucose and sucrose repression of Ac-CoA synthase and isocitrate lyase and to poor growth on sucrose-acetamide medium. This mutation is completely epistatic to the *amdI*9 mutation for growth on sucrose-acetamide medium (Kelly and Hynes, unpublished data).

It has been previously reported that β-hydroxypropionamide is a nonsubstrate (amide) inducer of the acetamidase (14). This observation appears to disagree with the proposal that amides must be metabolized to result in induction. However, this compound has been found to induce via a second induction pathway controlled by the *amdR* gene (called the *intA* gene by Arst [4]). This pathway of induction is more or less independent of induction by acetate and is effective in both the presence and absence of glucose (M. J. Hynes, unpublished data).

Mutations in yet another gene, *amdA*, also affect acetamidase regulation (4; Hynes, unpublished data). There is no compelling evidence for this gene being involved in induction by acetamide or acetate, but its role is not understood at present.

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