JOURNAL OF BACTERIOLOGY, Aug. 1970, p. 482-487
Vol. 103, No. 2
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Induction and Repression of Amidase Enzymes in *Aspergillus nidulans*

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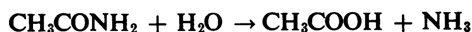
Received for publication 11 May 1970

Aspergillus nidulans can grow on acetamide as both a carbon and nitrogen source and can also grow on formamide as a nitrogen source. Two distinct enzymes, an acetamidase and a formamidase, are produced. The control of the synthesis of these two enzymes in a wild-type strain was investigated. The formamidase is induced by acetamide and formamide and repressed by ammonia. The acetamidase is induced by formamide and acetamide, repressed by carbon metabolites derived from glucose and acetate, and repressed by ammonia. Repression of the acetamidase by ammonia depends on the carbon source; growth on glucose but not on acetate or acetamide allows repression to occur. The pattern of acetamidase repression is compared with that of histidine catabolic enzymes in various bacteria.

Many catabolic enzymes in microorganisms are subject to repression by carbon metabolites. This phenomenon has been called catabolite repression (10). The best studied enzyme is β-galactosidase in *Escherichia coli*. This enzyme is repressed in cultures grown on glucose but not in cultures grown on glycerol (10, 15). In addition to catabolite repression of enzymes responsible for the utilization of carbon sources, repression of enzymes responsible for the utilization of nitrogen sources has been observed. In *Aspergillus nidulans* and *Neurospora crassa*, the nitrate reductase and nitrite reductase enzymes are repressed by ammonia (5, 7, 14). Similarly the enzymes of the pathway of purine degradation in *Aspergillus* are repressed by ammonia (6, 16).

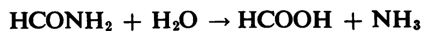
Some compounds are capable of acting as both carbon and nitrogen sources for microorganisms. The enzymes responsible for the utilization of these compounds can be repressed by either carbon or nitrogen metabolites, or both. If repression is only by carbon metabolites, the carbon source supplied can interfere with utilization of the compound as a nitrogen source; if repression is only by nitrogen metabolites, the nitrogen source can interfere with utilization of the compound as a carbon source. Therefore, if an organism is adapted to use a substance as either a carbon or a nitrogen source, its repression mechanisms must be adapted so that interference between carbon and nitrogen metabolism does not occur. The results of studies on histidine degradation in bacteria illustrate the above points. In *Bacillus subtilis* and *Salmonella typhimurium*, regulation of histidine degradation is by induction and by repression by carbon metabolites only (4, 11). In *Pseudomonas aeruginosa* and *Aerobacter aerogenes*, histidine degradation is subject to repression by both carbon metabolites and ammonia as well as to induction (8, 12).

This paper presents the results of studies on the regulation of amide utilization by amidase enzymes (acylamide amidohydrolase: EC 3.5.1.4) in *A. nidulans*. This organism can use acetamide as both a carbon source and a nitrogen source by means of an acetamidase enzyme. The reaction is



$$CH_3CONH_2 + H_2O \rightarrow CH_3COOH + NH_3$$

Formamide is used by *Aspergillus* as a nitrogen source only. Another enzyme, a formamidase, is involved in formamide utilization. The reaction is



$$HCONH_2 + H_2O \rightarrow HCOOH + NH_3$$

That the enzymes are separate and specific for their particular substrates has been shown by isolating mutants that lack the enzymes. Mutants lacking the acetamidase and unable to grow on acetamide have lesions which map at a different genetic locus from that of a mutant lacking the formamidase and unable to grow on formamide (M. J. Hynes and J. A. J. Pateman, *submitted for publication*). The results reported in this paper concern the induction and repression of the two amidase enzymes in the wild-type organism, and, in particular, the interaction between carbon

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and nitrogen metabolite control of the acetamidase.

MATERIALS AND METHODS

Strain. The strain used was biotin1, a biotin auxotroph originating from the Department of Genetics, University of Glasgow.

Media and supplements. Glucose media and supplements used were as described by Cove (5). Acetate-minimal medium was that described by Apirion (1) except that sodium acetate (12 g/liter) replaced ammonium acetate. When acetamide was used as a carbon source, the medium was again as described by Apirion (1), with acetamide at the desired concentration replacing acetate. Carbon-free medium was simply minimal salts solution. Acetamide and formamide were stored at 4 C as concentrated aqueous solutions and sterilized by Seitz filtration.

Growth tests. Growth testing was carried out at 37 C on solid medium (1.2% agar) in plastic petri dishes.

Growth of mycelium for enzyme assays. Mycelium was grown in shaken culture at 25 C, harvested, and extracted as described by Cove (5). All extracts were made in 0.1 M tris(hydroxymethyl)aminomethane-(Tris)-hydrochloride buffer (*p*H 7.2). When mycelium was pregrown before transfer to a special treatment medium (as in carbon and nitrogen starvation experiments), it was grown on glucose minimal medium usually with 5 mM urea as a nitrogen source for 18 to 19 hr, harvested by filtering through nylon net, washed with the treatment medium, and then transferred to the fresh medium. After the desired time of growth, the mycelium was harvested.

Protein determinations. The method of Lowry et al. (9) was used with serum albumin as a standard. The protein concentrations of the cell-free extracts were usually in the range of 1 to 2 mg per ml.

Enzyme assays. The same method was used for both the acetamidase and the formamidase. The basis of the assay was the determination of ammonia by the method described by Muftic (13). The phenol and the hypochlorite reagents were prepared as described (13). Substrate solutions were made up in 0.1 M pyrophosphate buffer (*p*H 7.2). Both formamide and acetamide were used at concentrations of 0.1 to 0.2 M. The substrate solution (1 ml) was placed in each of two test tubes. Cell-free extract (0.1 ml) was added to one of the tubes and incubated in a water bath at 37 C for the desired reaction time. The reaction was stopped with 0.5 ml of the phenol reagent, and then 1 ml of the hypochlorite solution was added. The second tube was a control, to which the phenol was added before the addition of cell-free extract; hypochlorite solution was added as in the first tube. A stable blue color developed within 10 min, and the absorbancy was measured at 655 nm. A curve relating ammonia concentration to absorbancy was constructed by using solutions of ammonium tartrate or ammonium chloride.

The assays were found to be linear with time and with the amount of extract used. All enzyme activities presented in this paper are expressed in nanomoles of ammonia released per minute per milligram of protein.

RESULTS

Table 1 shows the enzyme activities of extracts of mycelium grown on glucose minimal medium with different sources of nitrogen. Growth on ammonium tartrate gave repression of both the acetamidase and the formamidase enzymes. Growth on acetamide or formamide as the sole nitrogen source resulted in high enzyme activities, whereas growth on urea plus these amides gave somewhat lower activities but these were 1.5 to 3 times higher than on urea alone. Evidently, induction by the amides occurs but its level is reduced by the presence of other nitrogen sources. This was also shown by the finding that the strain *uZ4*, which lacks urease activity (16), when grown on urea plus acetamide, gave a similar acetamidase level to the activities obtained on acetamide alone. When ammonium and acetamide were the nitrogen sources, the enzymes were repressed. Repression by ammonia therefore prevented induction.

On limiting concentrations of urea (0.0125 M), where the urea was presumably exhausted before harvesting, the amidases were derepressed to levels higher than those of mycelium grown on a higher concentration of urea (5 mM). To study further the effects of the nitrogen status on enzyme activities, nitrogen starvation experiments were performed (Table 2). It was seen that the acetamidase was derepressed about twofold with starvation, whereas the formamidase was derepressed about threefold. The formamidase activities in fact approached those of mycelium grown on formamide alone.

These results showed that, on glucose medium, the amidase enzymes were controlled by induction by acetamide and formamide and by repression by nitrogen metabolites (probably ammonia, *see below*).

Table 3 shows the results of experiments in which mycelium was starved for carbon. The acetamidase was derepressed about fivefold under conditions of carbon starvation, whereas the formamidase was not significantly affected. This indicated that the acetamidase was subject to repression by the level of carbon metabolites. Table 4 shows that growth for 6 to 8 hr on acetamide as the sole source of carbon and nitrogen gave acetamidase levels tenfold higher than on glucose-urea medium and twofold higher than on carbon-free medium. Actidione, an inhibitor of protein synthesis, prevented the synthesis of the acetamidase. Under these conditions the formamidase initially increased, presumably due to acetamide induction; however, as acetamide utilization increased, formamidase levels dropped, probably as a result of repression by the ammonia released from acetamide.

TABLE 1. *Effect of the nitrogen source available for growth on amidase activities*

Nitrogen source[a]	Enzyme activity[b]	
	Acetamidase	Formamidase
0.005 M Ammonium tartrate	0	0
0.0125 M Urea	23	48
0.005 M Urea	15	25
0.005 M Urea + 0.02 M acetamide	26	39
0.0125 M Urea + 0.01 M acetamide	48	91
0.0125 M Urea + 0.01 M formamide	35	65
0.01 M Acetamide	103	132
0.01 M Formamide	90	109
0.005 M Ammonium tartrate + 0.01 M acetamide	6	0

[a] Added to glucose-minimal medium.
[b] Nanomoles of NH_4^+ per minute per milligram of protein.

TABLE 2. *Effect of nitrogen starvation on amidase activities*

Time of nitrogen starvation[a]	Enzyme activity	
	Acetamidase	Formamidase
hr		
0	15	31
1	18	41
2	25	27
3	32	60
4	35	92
5	28	89
6	27	104

[a] Time of incubation on nitrogen-free minimal medium after transfer from glucose-urea medium.

The results presented in Table 5 show that repression of the acetamidase by ammonia was dependent on the carbon source. When ammonia was the nitrogen source on carbon-free medium, the acetamidase activity was not repressed compared with the level on carbon-free medium with urea as the nitrogen source. When ammonia was the nitrogen source on acetamide medium, the acetamidase level was not repressed compared with the level on acetamide medium alone. This was supported by growth tests in which high concentrations of NH_4^+ did not inhibit growth on acetamide as a carbon source. When glucose was the carbon source, and acetamide and NH_4^+ the nitrogen sources, the acetamidase was repressed compared with the level on carbon-free medium with these nitrogen sources. Thus, the acetamidase was subject to ammonia repression only when glucose was the carbon source. Ammonia repression of the acetamidase was not evident when acetate was the carbon source. This was shown particularly by acetamidase derepression after transfer from glucose-NH_4^+ medium to acetate-NH_4^+ medium.

In contrast to the acetamidase, formamidase repression by ammonia did not appear to be dependent on the carbon source supplied. Formamidase levels were always low when ammonia was present. When acetamide as well as ammonia was present, the formamidase activities were somewhat higher, perhaps due to competition between induction by acetamide and repression by ammonia.

Table 5 also shows that acetate was a source of repressing carbon metabolites. The acetamidase activities on acetate-urea were much lower than on carbon-free medium; on acetate-acetamide medium, the acetamidase was lower than the level occurring when acetamide was the sole carbon source. Therefore, the effects of the carbon

TABLE 3. *Effect of carbon starvation on amidase activities*

Time of carbon starvation[a]	Enzyme activities	
	Acetamidase	Formamidase
hr		
0	20	39
2	59	53
4	73	46
5	72	47
6	82	35
8	100	

[a] Time of incubation on carbon-free minimal medium with urea (0.005 M) as nitrogen source after transfer from glucose-urea medium.

TABLE 4. *Effect of growth on acetamide as the sole source of carbon and nitrogen on amidase activities*

Time of growth on acetamide[a]	Enzyme activities	
	Acetamidase	Formamidase
hr		
2	49	62
4	98	34
6	159	36
8	172	20
6[b]	5	15

[a] Time of incubation on 0.1 M acetamide-minimal medium after transfer from glucose-urea medium.
[b] One-hundred-and-fifty micrograms of actidione added per ml of acetamide-minimal medium.

source on ammonia repression of the acetamidase could be separated from repression of the enzyme by carbon metabolites. Ammonia repression was dependent on glucose but not on acetate, whereas both glucose and acetate gave rise to carbon metabolite repression. This separation of the effects of the carbon source was also shown by the properties of a mutant derepressed for carbon metabolite repression but still subject to ammonia repression (Hynes and Pateman, *submitted for publication*).

When glutamate was the sole nitrogen source on glucose medium, the amidase levels were quite high. In contrast to this, glutamine gave rise to low levels of both enzymes (Table 5).

DISCUSSION

Synthesis of the formamidase enzyme appears to be controlled by induction by amides and repression by ammonia. The relative effect of repression appears to be large in comparison with the effect of induction. Derepression of the enzyme with nitrogen starvation leads to enzyme levels close to those of induced cultures. Carbon starvation does not significantly affect formamidase synthesis and the carbon source does not affect ammonia repression of the enzyme. This is consistent with formamide, a one-carbon compound, being a nitrogen source but not a carbon source.

Synthesis of the acetamidase enzyme is controlled by induction by amides, repression by carbon metabolites readily derived from glucose and acetate, and repression by ammonia. Ammonia repression of the acetamidase is dependent on the carbon source supplied; glucose but not acetate allows repression to occur. In the absence of a carbon source and when acetamide is the carbon source, ammonia repression is relieved. The net effect of these control mechanisms is to allow efficient utilization of acetamide as both a carbon source and as a nitrogen source. When acetamide is "required" as a nitrogen source, the combined effects of induction and derepression by absence of any other nitrogen source allow sufficient enzyme to be formed to permit growth. When acetamide is the sole carbon source, the nitrogen status does not affect enzyme synthesis, and the combined effects of derepression by absence of any other carbon source and induction by acetamide result in high enzyme levels. Thus, carbon metabolism does not interfere with use of acetamide as a nitrogen source and, conversely, ammonia repression does not interfere with the use of acetamide as a carbon source.

It is of interest to compare the control of the

TABLE 5. *Effect of various combinations of carbon and nitrogen sources for growth on amidase activities*

Medium[a]		Enzyme activities	
Carbon source	Nitrogen source	Acetamidase	Formamidase
	0.01 M NH_4^+ [b]	81	4
	0.005 M Urea	82	35
		46	64
	0.01 M Acetamide + 0.01 M NH_4^+	165	22
Glucose	0.01 M Acetamide + 0.01 M NH_4^+	14	13
0.1 M Acetamide		172	20
0.1 M Acetamide	0.01 M NH_4^+	196	19
Sodium acetate	0.005 M Urea	29	31
Sodium acetate	0.01 M NH_4^+	40	4
Sodium acetate	0.01 M Acetamide	104	97
Sodium acetate[c]	0.01 M NH_4^+	32	3
Glucose	0.01 M Glutamate	22	57
Glucose	0.01 M Glutamine	4	9

[a] Mycelium pregrown on glucose-urea medium for 18 to 19 hr and then transferred to the treatment medium for 6 to 8 hr before harvesting.

[b] Added as ammonium tartrate.

[c] Mycelium was pregrown on glucose—NH_4^+ medium before transferring to this treatment medium.

acetamidase in *Aspergillus* with the control of histidine catabolism in bacteria. In *B. subtilis* and *S. typhimurium*, the enzymes of histidine degradation are subject to repression by carbon metabolites but are not subject to repression by ammonia (4, 11); as a result, histidine is a very poor sole source of nitrogen on carbon sources, such as glucose, which are good sources of catabolite repression (i.e., carbon metabolism interferes with histidine nitrogen metabolism). In *P. aeruginosa* and *A. aerogenes*, the enzymes of histidine degradation are subject to carbon metabolite and ammonia repression (8, 12). Maximal enzyme activities occur when histidine is the sole source of carbon, and very low activities are observed when a repressing carbon source and ammonia are present. Histidine can act as the sole nitrogen source on medium with a repressing carbon source, and intermediate enzyme levels are observed. Thus, in these two organisms, the regulation mechanism is such that carbon metabolism does not affect the utilization of histidine as a nitrogen source. An entirely analogous situation appears to occur with acetamidase control in *Aspergillus*.

Acetamide can act as a carbon and nitrogen source for *P. aeruginosa*, and the control of the amidase enzyme responsible for acetamide utilization has been studied (3). The amidase is inducible

by amides and is repressed by carbon metabolites. The effects of ammonia on enzyme synthesis have not been reported.

Two types of hypothesis are possible to account for the dependence of ammonia repression of the acetamidase of *Aspergillus* on carbon metabolism. The first of these postulates that the true corepressing metabolite is a compound derived from both carbon and nitrogen metabolism. This type of hypothesis has been proposed by Lessie and Neidhardt to account for the repression of enzymes of histidine metabolism in *P. aeruginosa* (8). Low carbon status or low nitrogen status would result in some derepression due to reduction in the level of the repressing metabolite. Probably the principal pathway of ammonia assimilation into carbon compounds is via glutamate formation by glutamate dehydrogenase. High levels of this enzyme occur when bacteria are grown on glucose and ammonia (12). Thus, a likely candidate for a combined carbon-nitrogen corepressor would be glutamate or a derivative thereof. However, when glutamate is the sole nitrogen source, the acetamidase is not repressed, and it is known that there is a high glutamate pool when *Aspergillus* is grown on glutamate as a sole nitrogen source (Pateman, *manuscript in preparation*). The data above suggest that the first hypothesis is rather unlikely; in addition, there is evidence which suggests that ammonia itself is a corepressor. Arst and Cove have shown that, in a number of repressible pathways in *Aspergillus*, methyl-ammonium acts as an analogue of ammonia in repression, and this occurs in a mutant unable to use methyl-ammonium as a nitrogen source (2). In particular a growth test indicated that methyl-ammonium inhibited the use of acetamide as a nitrogen source. It is therefore necessary to propose that, if ammonia itself is not the corepressor, the corepressor can be formed from both methyl-ammonium and ammonia. Methyl-ammonium is probably not a substrate, at least in vitro, for the glutamate dehydrogenase of *Aspergillus* (Pateman, *unpublished data*). Evidence has indicated that the formamidase and the acetamidase are subject to repression by the same nitrogen metabolite (Hynes and Pateman, *submitted for publication*). Mutation in a regulatory gene can lead to both enzymes being supersensitive to repression. Since the repression of the formamidase is not affected by the carbon source this makes this first hypothesis more unlikely.

A second proposal is that repression of the acetamidase is concerted. Two compounds act as corepressors, one a nitrogenous metabolite (ammonia) and the other a carbon metabolite. Full repression would only be possible when both corepressors are present. The mechanism of action would be analogous to those involved in the repression and feedback inhibition mechanisms operating in the control of some amino acid biosynthetic pathways (17). There is no evidence for or against this hypothesis at present.

Both amidase enzymes do not appear to be under strict control in that considerable levels of the enzymes may be present in cultures in the absence of an inducer-substrate (e.g., during starvation experiments). This results from the great importance of repression in control of the enzymes. Nitrate assimilation in *Aspergillus* appears to have a much stricter requirement for induction. In a preliminary experiment no derepression of nitrate reductase occurred with nitrogen starvation.

Thus, the control of formamidase appears to be by ammonia repression and amide induction. The acetamidase is controlled by amide induction, combined repression by ammonia and a carbon metabolite readily derived from glucose, but not acetate, and repression by a carbon metabolite readily derived from glucose and acetate. Genetic studies of amidase regulation will be reported elsewhere (Hynes and Pateman, *submitted for publication*).

ACKNOWLEDGMENTS

The help and advice of J. A. Pateman is greatly appreciated.

This work was supported by a Commonwealth Postgraduate Scholarship.

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