SHORT COMMUNICATION

Genetic Evidence for a Second Asparaginase in *Aspergillus nidulans*

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(Received 19 May 1980)

The *apnA1* mutation strongly reduces L-asparagine utilization in *Aspergillus nidulans*. The *ahrA1* mutation, leading to loss of an L-asparaginase (Drainas *et al.*, 1977), eliminates residual L-asparagine utilization in double mutant strains also carrying *apnA1*. This additivity suggests that *A. nidulans*, like *Saccharomyces cerevisiae* (Jones, 1977; Dunlop *et al.*, 1978), has two L-asparaginases specified by *apnA* and *ahrA*, respectively. *apnA* has been mapped to a position on the left arm of linkage group II, in the sequence *adH-acrA-apnA-wA-methA-palcA* (centromere).

INTRODUCTION

Drainas *et al.* (1977) reported the selection of mutations for resistance to the toxicity of DL- β -aspartylhydroxamate in the ascomycete fungus *Aspergillus nidulans*. The mutations mapped in a gene designated *ahrA* and led to loss of L-asparaginase (L-asparagine amidohydrolase; EC 3.5.1.1) activity. They concluded that the toxicity of DL- β -aspartylhydroxamate to *A. nidulans* results from hydroxylamine produced upon its hydrolysis, catalysed by the L-asparaginase specified by the *ahrA* gene. They showed, however, that *ahrA* mutants, although lacking L-asparaginase activity, utilize L-asparagine as a carbon or nitrogen source as well as the wild-type. A possible clue for the resolution of this apparent paradox was provided by the finding that the yeast *Saccharomyces cerevisiae* synthesizes two genetically and biochemically distinct L-asparaginases (Jones, 1977; Dunlop *et al.*, 1978 and references therein). This paper reports the selection of a mutation, designated *apnA1*, which reduces L-asparagine utilization in *A. nidulans*. *apnA1* is additive with *ahrA1*, resulting in complete inability to utilize L-asparagine in *apnA1 ahrA1* double mutants, thus suggesting that it abolishes a second L-asparaginase. Because this work falls outside our main research interests, we have not attempted biochemical characterization of the *apnA1* mutation. Nevertheless, this brief report of our results should interest readers studying asparaginases in *A. nidulans* and other organisms.

METHODS

Chemicals. Chemicals used were of the highest grade commercially available. D-Asparagine, D-glutamine, DL- β -aspartylhydroxamate and L- γ -glutamylhydroxamate were purchased from Sigma.

Strains and genetic techniques. Genetic techniques were modified after Pontecorvo *et al.* (1953), McCully & Forbes (1965) and Clutterbuck (1974). Markers carried by *A. nidulans* strains have been described previously (Clutterbuck, 1974; Arst, 1976; Drainas *et al.*, 1977; Arst *et al.*, 1978) with the exception of *apnA1*

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whose selection is described in this paper. We are grateful to Dr J. R. Kinghorn and Professor J. A. Pateman for providing the *ahrA1* marker and to Dr A. Paszewski for providing the *methA17* marker.

Growth tests. These have been described previously (Arst & Cove, 1969). The minimal medium described by Cove (1966) was used in all growth tests. Except where a different carbon source is specified, 1% (w/v) D-glucose served as carbon source. Amino acids were used as nitrogen sources at 5 mM and as carbon sources at 50 mM. When amino acids served as carbon sources, 10 mM ammonium (chloride) was present as an additional nitrogen source. Nitrate (as the sodium salt), ammonium [as the (+)-tartrate], acetamide and acrylamide were used as nitrogen sources at 10 mM. Growth tests were scored after 2 or 3 d incubation at 37 °C. All growth scores and characteristics reported are based on examination of at least 50 (and frequently more) independent strains having the relevant partial genotype specified.

Selection of *apnA1*. *apnA1* was selected in the course of an experiment designed to yield mutants altered generally in the control of nitrogen metabolism. A strain of genotype *pabaA1 fwA1* (*p*-aminobenzoate-requiring, having fawn conidial colour) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Alderson & Hartley, 1969) and plated on appropriately supplemented minimal medium (Cove, 1966) containing 1% (w/v) D-glucose as carbon source, 10 mM-acrylamide as nitrogen source and 500 µM-DL-β-aspartylhydroxamate and incubated at 37 °C. Wild-type strains such as the parent are unable to grow on this medium because (i) they cannot utilize acrylamide as nitrogen source (Hynes & Pateman, 1970) and (ii) they are sensitive to DL-β-aspartylhydroxamate toxicity (Drainas *et al.*, 1977). From a number of mutants able to grow on this medium, one was chosen for further study because it had both gained the ability to utilize acrylamide as nitrogen source and largely lost the ability to utilize L-asparagine (but no other compound screened) as carbon and/or nitrogen source. Upon outcrossing, these two properties segregated independently.

RESULTS AND DISCUSSION

The ability of the original mutant strain to utilize acrylamide probably results from an *intA^c* mutation because it has the following additional pleiotropic effects. (1) It enhances utilization of 2-pyrrolidone as carbon and/or nitrogen source and 2-piperidone as nitrogen source (Arst *et al.*, 1978). (2) It results in resistance to the toxicities of a number of growth inhibitors such as Cs⁺, ClO₃⁻, methylammonium, DL-β-aspartylhydroxamate and thiourea, whose toxicities can be reversed by exogenous ammonium, in the presence of γ-amino-*n*-butyrate or acetamide as nitrogen source but not in the presence of L-proline or most other nitrogen sources. Resistance to such inhibitors on a particular nitrogen source is characteristic of regulatory mutations which enhance catabolism of that nitrogen source (Arst & Cove, 1973; Bailey & Arst, 1975; Bailey *et al.*, 1979; Arst *et al.*, 1980; see also Rand & Arst, 1977). Resistance to such inhibitors on γ-amino-*n*-butyrate and acetamide is characteristic of *intA^c* mutations (Arst & Cove, 1973; Arst, 1976; H. N. Arst, Jr & C. R. Bailey, unpublished results). However, as this putative *intA^c* mutation is clearly not responsible for the decrease in L-asparagine utilization, it was not investigated further.

The apparently simultaneous selection of a mutation, designated *apnA1*, reducing utilization of L-asparagine but not any other of a wide range of carbon and/or nitrogen sources tested might have been fortuitous, as the mutation confers no apparent advantage on the selection medium. The putative *intA^c* mutation results not only in ability to utilize acrylamide as nitrogen source but apparently also in some resistance to DL-β-aspartylhydroxamate (as well as to Cs⁺ and other inhibitors of the group whose toxicities can be reversed by exogenous ammonium) in the presence of acrylamide. *apnA1* neither results in resistance to DL-β-aspartylhydroxamate on acrylamide as nitrogen source nor enhances growth of strains also carrying the putative *intA^c* mutation on the selection medium.

Phenotype of apnA1

apnA1 prevents utilization of L-asparagine as a carbon source and considerably reduces its utilization as a nitrogen source (Table 1). This effect appears to be confined to L-asparagine catabolism. In particular, *apnA1* does not affect utilization of L-glutamine, L-aspartate or L-glutamate as carbon sources nor utilization of D-asparagine, D- or L-glutamine, L-aspartate, L-glutamate or ammonium as nitrogen sources. Unlike *ahrA* mutations (Drainas

Table 1. *Growth responses of wild-type and mutant strains of A. nidulans*

Growth scores range from — (no growth) to $\pm$ (slight growth) to + to ++++ (increasing levels of growth) but are not necessarily equivalent on different media. Nitrogen source utilization was tested using solid minimal medium (Cove, 1966) containing 1% (w/v) D-glucose as carbon source and scored after 2 d incubation at 37 °C. Carbon source utilization was tested using glucose-free solid minimal medium (Cove, 1966) containing 10 mM-ammonium (chloride) as nitrogen source and scored after 3 d incubation at 37 °C.

Relevant genotype	Growth on media containing:			
	50 mm-L-asparagine as C	5 mm-L-asparagine as N	2.5 mm-N-acetyl-L-asparagine as N	5 mm-D-asparagine as N
Wild-type	++	++++	++	++
<i>apnA1</i>	—	+	++	++
<i>ahrA1</i>	++	++++	$\pm$	+
<i>apnA1 ahrA1</i>	—	—	—	+

et al., 1977) and a class of possible asparagine/glutamine transport mutations (Kinghorn & Pateman, 1977), *apnA1* does not affect toxicity of DL- β -aspartylhydroxamate when tested on a range of nitrogen sources including L-alanine, L-glutamate, γ -amino-*n*-butyrate, L-proline, acetamide, acrylamide and nitrate. Unlike the possible asparagine/glutamine transport mutations and a putative glutaminaseless mutation (Kinghorn & Pateman, 1977), *apnA1* does not affect toxicity of L- γ -glutamylhydroxamate (tested on L-alanine and nitrate as nitrogen sources).

apnA1 is recessive in heterozygous diploids for utilization of L-asparagine as a nitrogen source but not completely recessive when tested for its utilization as a carbon source. Such a gene dosage effect has also been demonstrated for *ahrA* mutations (Drainas *et al.*, 1977). Supplementation of the L-asparagine auxotrophy *asnA68* (Gunatilleke & Arst, as cited in Arst & Page, 1973) is not affected by *apnA1* in *apnA1 asnA68* double mutants.

Whilst having no apparent effect on L-asparagine utilization on its own, *ahrA1* eliminates residual L-asparagine utilization in *ahrA1 apnA1* double mutants (Table 1). This additivity suggests that *A. nidulans*, like *S. cerevisiae*, has two L-asparaginases specified by the *ahrA* and *apnA* genes, respectively. The fact that *ahrA* mutations alter growth responses to DL- β -aspartylhydroxamate (Drainas *et al.*, 1977), N-acetyl-L-asparagine and D-asparagine (Table 1) might indicate that the enzyme specified by *ahrA* is somewhat similar to asparaginase II of *S. cerevisiae* (Dunlop *et al.*, 1978, 1980*a*).

Although the existence of a second L-asparaginase specified by the *apnA* gene would explain why *ahrA* mutants utilize L-asparagine, it is curious that this activity was not detected, at least in extracts of *ahrA* mutants, by Drainas *et al.* (1977). One possible explanation is suggested by the wild-type growth response of *apnA1* strains to DL- β -aspartylhydroxamate, namely, that the enzyme specified by *apnA* might not catalyse the hydroxyaminolysis reaction (which would yield L- β -aspartylhydroxamate as a reaction product). If, as tentatively suggested by the growth data, the *apnA*-specified enzyme cannot catalyse the hydrolysis of L- β -aspartylhydroxamate, it is probably also unable to catalyse the hydroxyaminolysis of L-asparagine, the reaction used by Drainas *et al.* (1977) to assay L-asparaginase activity. These authors do state that their observed hydroxyaminolysis activity correlated well with measurements of hydrolysis activity (yielding L-aspartate and ammonium) but give no indication of how extensive their hydrolysis assays were. Another possible explanation is that different growth conditions might be necessary to detect the *apnA*-specified enzyme.

Map position of *apnA1*

Haploidization analysis (McCully & Forbes, 1965; Clutterbuck, 1974) located *apnA1* to linkage group II [whereas *ahrA* is located in linkage group VIII (Drainas *et al.*, 1977)]. Analysis of 618 progeny from a cross of relevant partial genotype *methA17* × *wA3 apnA1 acrA1 adH23* established the following linkage relationships on the centromere distal region of the left arm of linkage group II (distances in centimorgans):

As this *wA-methA* gene order disagrees with that reported previously (Gajewski & Litwińska, 1968; Putrament *et al.*, 1971) but was based on analysis of only eight recombinants between *wA3* and *methA17*, an additional 96 *wA⁺ methA⁺* recombinant progeny were selected and analysed: 13 carried *apnA1* (of which 12 also carried *acrA1*) and 16 carried *acrA1*, thus supporting the map order shown above. Finally, an additional cross was analysed in which *palcA1*, which is 19 cM centromere proximal to the *wA* gene (Clutterbuck, 1974; Arst *et al.*, 1979), was included and in which *wA3* and *methA17* were in coupling. The relevant partial genotype was *palcA1* × *methA17 wA3 apnA1 acrA1 adH23*, and 186 *wA3 methA⁺* progeny (accounting for 1.8% of the total *methA⁺* progeny) were selected. Of these 186 progeny, 38 were *palcA⁺*, 26 were *apnA⁺* (of which 23 were also *acrA⁺*), and 42 were *acrA⁺*, confirming the order: (*adH*)–*acrA*–*apnA*–*wA*–*methA*–*palcA*–(centromere). The basis for the disagreement of *wA-methA* gene order with that reported by the Warsaw group is unclear but of some importance because the gene order shown above would necessitate reinterpretation of the results presented by Putrament *et al.* (1971).

Note added in proof. A paper on the regulation of asparaginase II synthesis in *S. cerevisiae* has just appeared (Dunlop *et al.*, 1980b). The enzyme specified by *ahrA* of *A. nidulans* resembles yeast asparaginase II in regulation of its synthesis (see Drainas *et al.*, 1977) as well as in its inferred substrate specificity: neither enzyme is inducible by L-asparagine but both are subject to nitrogen metabolite repression.

We are grateful to the Royal Society for the Smithson Research Fellowship (H.N.A.) and to the Science Research Council for grants to Professor J. M. Thoday (C. R. B. and H.N.A.) and H.N.A. We thank Liz Workman and Shelagh Cousen for technical assistance and Olwen Fay for typing the manuscript.

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