NAD and NADP L-Glutamate Dehydrogenase Activity and Ammonium Regulation in *Aspergillus nidulans*

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SUMMARY

Mutants, designated *gdhA*, deficient in NADP-glutamate dehydrogenase activity grow poorly using inorganic nitrogen but normally using amino acids as nitrogen sources. The *gdhA* mutants are not repressed by ammonia for a number of enzyme and uptake systems. The nine *gdhA* mutations are allelic and located in linkage group III; *gdhA1* is recessive in the heterozygous diploid with respect to both NADP glutamate dehydrogenase activity and ammonium regulation.

Mutants, designated *gdhB*, deficient in NAD-glutamate dehydrogenase activity are unable to utilize L-glutamate as a carbon source, but can utilize inorganic and organic nitrogen as nitrogen sources. The four *gdhB* mutations are allelic, located in linkage group IV, and *gdhB1* is recessive in the heterozygous diploid. The *gdhB* mutants are repressed for the ammonium regulated systems which are not repressed in *gdhA* mutants.

INTRODUCTION

Aspergillus nidulans can synthesize two L-glutamate dehydrogenases. L-Glutamate:NADP oxidoreductase EC. 1.4.1.4 (NADP-GDH) has an aminating function and maximal activity is found in cells utilizing inorganic nitrogen and minimal activity in cells utilizing L-glutamate (Pateman, 1969). L-Glutamate:NAD oxidoreductase EC. 1.4.1.2 (NAD-GDH) has a deaminating function and optimal activity is found in cells utilizing L-glutamate as the sole carbon source (Kinghorn & Pateman, 1972). A similar situation is known to occur in a number of other higher fungi (LéJohn, 1971).

Grenson & Hou (1972) have described a mutant in *Saccharomyces cerevisiae* which lacks NADP-GDH activity and is abnormal with respect to the regulation by ammonium of the general amino acid permease. Mutations in a number of loci in *Aspergillus nidulans* can affect the regulation of a number of systems by ammonium. These systems include nitrate reductase (Pateman & Cove, 1967), hypoxanthine dehydrogenase (Scazzocchio & Darlington, 1968), extracellular protease (Cohen, 1972), acetamidase and formamidase (Hynes & Pateman, 1970*a, b*), glutamate uptake (Kinghorn & Pateman, 1972; Pateman & Kinghorn, unpublished) and urea uptake (Dunn & Pateman, 1972). Arst & Cove (1969) found that mutation in two loci *meaA* and *meaB* resulted in resistance to methylamine and derepression for many ammonium repressed systems. Cohen (1972) isolated a mutant *xprD1* which was derepressed for extracellular protease release and other systems. J. Pateman and others (unpublished) have isolated other classes of derepressed mutants, DER3 and *amrA1*, which have ammonium transport abnormalities. We report the level of NADP-GDH activity in the various classes of ammonium derepressed mutants. We also describe some properties of mutants deficient in either NADP-GDH or NAD-GDH activity, with particular reference to ammonium regulation.

METHODS

Materials. All reagents were obtained from commercial sources and were used without further purification.

Media and supplements. Medium used for genetic analysis was essentially that described by Pontecorvo, Roper, Hemmons, MacDonald & Bufton (1953). Nitrogen-less minimal medium (–N medium) (Cove, 1966), and carbon- and nitrogen-less minimal medium (–CN medium) were used. The nitrogen and carbon sources used in media were kept as sterile stock solutions at 1 M.

Growth of mycelium for enzyme assays. Cells were grown in shaken flask culture at 30 °C, harvested and extracted by grinding in a cold mortar essentially as described by Cove (1966). Mycelium was grown on –N medium containing 0.15 % Casamino acids plus 5 mM-ammonium tartrate for 15 h before transfer to treatment media for 5 h. For maximum NADP-GDH activity the treatment medium was –N medium plus 5 mM-ammonium tartrate. For maximum NAD-GDH activity the treatment medium was –CN medium plus 100 mM-glutamate. The mycelium was pressed dry, using paper towels, and weighed. Yields were usually 5 to 8 g/l medium.

L-Glutamate dehydrogenase. NAD-GDH and NADP-GDH were assayed following the reductive amination of α -oxoglutarate in the presence of ammonia and reduced NAD or NADP. The initial reaction velocity was estimated from the change in optical density at 340 nm in a Unicam SP 800 spectrophotometer. The reaction mixture for the NADP-GDH assay was: 0.4 M-NH₄Cl in 0.05 M-phosphate buffer (pH 7.75), 0.4 ml; 0.2 M- α -ketoglutarate in 0.05 M-phosphate buffer (pH 7.75), 0.2 ml; reduced NADP (2 mg/ml), 0.2 ml; 0.05 M-phosphate buffer (pH 7.75), 2 ml; enzyme extract (1 to 2 mg protein/ml), 0.2 ml. The reaction mixture for the NAD-GDH was: 0.4 M-NH₄Cl in 0.05 M-phosphate buffer (pH 8.0), 0.4 ml; 0.2 M- α -ketoglutarate in 0.05 M-phosphate buffer (pH 8.0), 0.2 ml; reduced NAD (2 mg/ml), 0.2 ml; 0.05 M-phosphate buffer (pH 8.0) with 0.1 mM- β -mercaptoethanol and 0.5 mM-EDTA. The assay temperature in both cases was 37 °C and the results expressed as nanomole substrate transformed/min/mg protein.

Plate tests for ammonium derepression. (1) Nitrate reductase: ammonium, by repressing nitrate reductase synthesis, protects the wild-type against chlorate toxicity; poor growth on –N medium plus 100 mM-KClO₃ plus 10 mM-ammonium indicates derepression of nitrate reductase (Arst & Cove, 1969). (2) Xanthine dehydrogenase: a green-spored wild-type develops yellow conidia in the presence of 2-thioxanthine due to the action of xanthine dehydrogenase. Ammonium prevents the development of yellow conidia by repressing xanthine dehydrogenase. The production of yellow conidia by a genotypically green-spored strain grown on –N medium plus 0.5 mM-thioxanthine plus 10 mM-ammonium, indicates derepression of xanthine dehydrogenase (Scazzocchio & Darlington, 1968). (3) Extracellular protease: ammonium derepression of protease release is indicated by a milk clearing halo around a colony growing on turbid –N medium plus milk plus 10 mM-ammonium (Cohen, 1972).

Plate test for methylamine resistance. Wild-type cells will not grow on –N medium plus 100 mM-methylamine chloride; methylamine resistant strains grow well (Arst & Cove, 1969).

Growth of mycelium for uptake assays. After growth in shaken flasks at 25 °C for 16 to 20 h on –N medium plus 0.15 % Casamino acids, cells were harvested and transferred to –N medium + 10 mM-ammonium tartrate for 4 h at 25 °C. In the latter treatment, cells were transferred five times during the 4 h period to maintain the ammonia concentration close to the original value. Finally, the cells were harvested and assayed.

L-Glutamate and thiourea uptake assays. When grown under the conditions described above, *Aspergillus nidulans* is largely in the form of small colonies less than 1 mm in diameter. These colonies can be kept in suspension by shaking and samples withdrawn quanti-

tively from the suspension. After growth and subsequent transfer to treatment medium for 5 h, 1 g fresh weight of the cells is resuspended in 50 ml of $-N$ medium plus L-glutamate or thiourea in a 250 ml Erlenmeyer flask in a shaking water bath at 25 °C. To assay L-glutamate uptake the flasks contain [^{12}C]L-glutamate and [^{14}C]L-glutamate to a final concentration of 100 μM and approximately 1.0 μC radioactivity; for thiourea uptake they contain [^{12}C]thiourea and [^{14}C]thiourea to a final concentration of 200 μM and approximately 3.0 μC radioactivity. At 2 min intervals from 2 to 10 min, 5 ml aliquots of the cell suspension are filtered with two washes of 10 ml of water, on a Millipore filter. The resultant pad of cells is transferred to 5 ml Bray's scintillation fluid and the radioactivity measured in a Beckman Liquid Scintillation Spectrometer. The rate of uptake of radioactivity into cells is linear for both substances up to 10 min. The level of uptake of the two substances in wild-type cells is 20 times higher on $-N$ medium treatment (derepressed level) than on $-N$ medium plus 10 mM-ammonium tartrate (repressed level). The results are expressed in terms of repressed or derepressed level in mutant cells after treatment on $-N$ medium with 10 mM-ammonium tartrate.

Strains and genetic analysis. The genetic techniques employed were those described by Pontecorvo *et al.* (1953) and McCully & Forbes (1965). The strains used were a biotin auxotroph *bi1* (Glasgow No. 051) and a biotin and putrescine auxotroph *bi1 puA2* (Glasgow No. 0171). *meaA8* and *meaB6* (formerly *meaA6*) are methylammonium-resistant, ammonium-derepressed mutants (Arst & Cove, 1969) supplied by Dr H. N. Arst. DER3 is one of a series of ammonium derepressed mutants obtained by J. A. Pateman selecting directly for ammonium derepression of nitrate reductase using a replica plating technique similar to that described by Pateman & Cove (1967). *amrA1* was obtained by J. A. Pateman selecting for mutants with poor growth on ammonium as a nitrogen source. *xprD1* is an ammonium derepressed mutant obtained by selecting directly for ammonium derepression of extra-cellular protease production (Cohen, 1972) supplied by Dr B. L. Cohen.

Protein determination. All protein determinations were carried out by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

Selection of NAD-GDH mutants. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treated conidia (Adelberg, Mandel & Chen, 1965) of *bi1* were spread on $-N$ medium with 2 mM-urea, biotin and sodium deoxycholate (Mackintosh & Pritchard, 1963). After incubation, the colonies were replicated on to $-N$ medium containing biotin and 100 mM-ammonium tartrate. Colonies which failed to grow on the 100 mM-ammonia medium were isolated and cloned by replating for isolated colonies.

Selection of mutants with low NAD-GDH activity. The putrescine technique (Herman & Clutterbuck, 1966) was used to isolate mutants unable to use L-glutamate as a sole carbon and nitrogen source. Clumps of NTG-treated conidia of *bi1 puA2* were inoculated on to solid $-NC$ medium plus biotin, limiting putrescine and with 100 mM-L-glutamate as sole carbon and nitrogen source. Sectors showing spidery growth were isolated. A number of these proved to be mutants unable to utilize L-glutamate.

RESULTS

Isolation and characterization of mutants

Nine mutants, designated *gdhA1* to *gdhA9*, were isolated on the basis of sensitivity to high concentrations of ammonium (ammonium-sensitive mutants). Diploids between *gdhA1* and the other *gdhA* mutants, and heterokaryons of all possible combinations of *gdhA* mutants, were made. The mutants were shown to be allelic by their failure to complement in these

Table 1. *NADP and NAD-GDH activities in *gdhA*, *gdhB* and various classes of ammonium derepressed mutants*

The mycelia were grown and treated as described in the Methods section to give the maximum NADP-GDH or NAD-GDH activity

Strain	Enzyme activity (nmole/min/mg)	
	NADP-GDH	NAD-GDH
Wild-type	1940	1520
<i>gdhA1</i>	*	1610
<i>gdhA2</i>	*	1600
<i>gdhA3</i>	*	1300
<i>gdhA4</i>	*	1490
<i>gdhA5</i>	310	1590
<i>gdhA6</i>	350	1210
<i>gdhA7</i>	320	1430
<i>gdhA8</i>	210	1630
<i>gdhA9</i>	395	1690
<i>gdhA1</i> /+	1810	1630
<i>gdhB1</i>	1830	*
<i>gdhB2</i>	2015	*
<i>gdhB3</i>	2010	*
<i>gdhB4</i>	1895	*
<i>gdhB1</i> /+	1825	1200
<i>meaA8</i>	1790	N.T.
<i>meaB6</i>	1520	N.T.
<i>xprD1</i>	1840	N.T.
<i>amrA1</i>	2050	N.T.
DER3	1615	N.T.

N.T. Not tested. * No detectable activity.

diploids and heterokaryons. The *gdhA* locus was assigned to the *argB-galD* interval of the III linkage group. The *gdhA* locus shows 50 % recombination with *meaA6* and 24 % with *xprD1*, which are in the same linkage group.

Four mutants, *gdhB1*–*gdhB4*, were isolated, which were unable to utilize L-glutamate as a carbon source. Similarly they were found to be allelic by complementation tests carried out on the heterokaryons. The *gdhB* locus was assigned to linkage group IV.

*Glutamate dehydrogenase activity in *gdhA* and *gdhB* mutants*

It can be seen from Table 1 that *gdhA* mutations lead to decreased activities of NADP-GDH, from zero to 20 % of wild-type, while NAD-GDH is unaffected. The heterozygous diploid *gdhA1*/+ has similar NADP-GDH activity to the haploid *gdhA*⁺, indicating that the *gdhA1* mutation is recessive.

Conversely, *gdhB* mutants have undetectable NAD-GDH activity and wild-type NADP-GDH levels. The heterozygous diploid *gdhB1*/+ has similar NAD-GDH activity to the haploid *gdhB*⁺, indicating that the *gdhB1* mutation is recessive.

The NADP-GDH levels of the wild-type and the derepressed mutants *meaA8*, *meaB6*, *xprD1*, *amrA1* and DER3 are given in Table 1. The only class of derepressed mutants which has low NADP-GDH is the ammonium-sensitive class, the *gdhA* mutants.

Table 2. *The level of some enzyme and uptake systems which are regulated by ammonium in the *gdhA* and *gdhB* mutants*

Ammonium repression of xanthine dehydrogenase, nitrate reductase and extracellular protease was determined by the plate tests given in Methods. Ammonium repression of L-glutamate uptake was determined by L-glutamate uptake assays. Ammonium repression of thiourea uptake was determined by thiourea uptake assays and the thiourea plate test given in Methods.

Strain	Xanthine dehydrogenase	Nitrate reductase	Extracellular protease	Glutamate uptake	Thiourea uptake
Wild-type	R	R	R	R	R
<i>gdhA1</i>	D	D	R	D	D
<i>gdhA2</i>	D	D	R	D	D
<i>gdhA3</i>	D	D	R	D	D
<i>gdhA4</i>	D	D	R	D	D
<i>gdhA5</i>	D	D	R	D	D
<i>gdhA6</i>	D	D	R	D	D
<i>gdhA7</i>	D	D	R	D	D
<i>gdhA8</i>	D	D	R	D	D
<i>gdhA9</i>	D	D	R	D	D
<i>gdhA1</i> /+	R	R	R	R	R
<i>gdhB1</i>	R	R	R	R	R
<i>gdhB2</i>	R	R	R	N.T.	N.T.
<i>gdhB3</i>	R	R	R	N.T.	N.T.
<i>gdhB4</i>	R	R	R	N.T.	N.T.

R, System repressed by ammonia; D, system *not* repressed by ammonia; N.T. not tested.

Table 3. *Growth responses of *gdhA1* and *gdhB1* mutants on certain nitrogen and carbon sources*

Genotypes	- N medium (solid)							
	10 mM-ammonia	10 mM-NO ₃	10 mM-urea	10 mM-L-glutamate	10 mM-L-alanine	10 mM-L-aspartate	100 mM-urea	100 mM-ammonia
Wild-type	+	+	+	+	+	+	+	+
<i>gdhA1</i>	±	±	±	+	+	+	—	—
<i>gdhA1</i> /+	+	+	+	+	+	+	+	+
<i>gdhB1</i>	+	+	+	+	+	+	+	+
<i>gdhB1</i> /+	+	+	+	+	+	+	+	+

Genotypes	- CN medium (solid)	
	100 mM-glutamate	100 mM-glutamate + 50 mM-ammonia
Wild-type	+	+
<i>gdhA1</i>	±	—
<i>gdhA1</i> /+	+	+
<i>gdhB1</i>	—	—
<i>gdhB1</i> /+	+	+

+ Normal growth of wild-type; ± up to 50 % growth of that of wild-type; — extremely poor growth.

*The *gdhA* mutation and ammonium derepression*

The results given in Table 2 show that *gdhA* mutations result in the failure of ammonium to repress xanthine dehydrogenase, nitrate reductase, glutamate uptake and urea uptake, but the mutants are repressed for protease. The heterozygous diploid *gdhA1*/+ is repressed for all of these systems. The *gdhB* mutants are also repressed.

*Growth responses of *gdhA* and *gdhB* mutants*

Mutation in the *gdhA* locus results in poor growth on inorganic nitrogen sources. In addition, the *gdhA* mutants are sensitive to high concentrations of ammonium and grow very poorly in the presence of 100 to 200 mM-ammonium or 100 mM-urea. With 50 mM-L-glutamate as the sole carbon source, the *gdhA* mutants grow less than the wild-type. *gdhA* mutants grow very poorly with L-glutamate as the carbon source together with 50 mM-ammonium. The heterozygous diploid, *gdhA1*/+, grows like the haploid wild-type in all the test conditions.

The *gdhB* mutants grow as well as the wild-type on all nitrogen sources. The *gdhB* mutants grow poorly on L-glutamate as the sole carbon source. The heterozygous diploid, *gdhB1*/+, grows as well as the haploid wild-type with L-glutamate as the sole carbon source.

DISCUSSION

Aspergillus nidulans produces two types of glutamate dehydrogenase; one form specific for NAD has a degradative deaminating function, the other specific for NADP has a synthetic aminating function. The different *in vivo* functions of the two enzymes are clearly demonstrated by the conditions which determine the synthesis of the two enzymes and the growth properties of strains carrying mutations at the *gdhA* and *gdhB* loci. The synthesis of NADP-GDH in *A. nidulans* is probably regulated by L-glutamate (Pateman, 1969). The synthesis of NAD-GDH in *A. nidulans* is probably regulated by glucose or some metabolite derived from glucose J. R. (Kinghorn & J. A. Pateman, unpublished). The *gdhA* mutants, which lack NADP-GDH activity, are unable to utilize inorganic nitrogen efficiently for the synthesis of glutamic acid and require a supplement of amino acid for normal growth. In the presence of the usual carbon sources the synthesis of NAD-GDH does not fully substitute for the lack of NADP-GDH. The *gdhA* mutants grow poorly when L-glutamate is the sole carbon source, despite the production of NAD-GDH, but this is probably a secondary effect which is discussed below. The *gdhB* mutants, which lack NAD-GDH activity, can utilize inorganic nitrogen and do not require an amino acid supplement for normal growth. The *gdhB* mutants are unable to utilize L-glutamate as a carbon source, since the glutamate repressed level of NADP-GDH is insufficient for the deamination of glutamate.

The *gdhA* mutants are sensitive to high concentrations of ammonium. The reason for this is not clear. It may be connected with the fact that *gdhA* mutants have a higher intracellular ammonium concentration than the wild-type in the presence of extracellular ammonium or urea. However, some other ammonium derepressed mutants also have high intracellular ammonium pools, but are not ammonium-sensitive (J. A. Pateman and others, unpublished). It is probable that the poor growth of *gdhA* mutants on L-glutamate as a carbon source is due to the effect of the large amounts of ammonium produced from the glutamate by the NAD-GDH. The *gdhB* mutants are not sensitive to high concentrations of ammonium.

The *gdhA* and *gdhB* loci are probably the structural genes for the NADP-GDH and NAD-GDH enzymes respectively, since no other loci are known at which mutation can result in

the lack of either activity. In addition to the differences in coenzyme specificity, function, regulation and genetic determination, there is a further important distinction between the two forms of GDH. The *gdhA* mutants, but not the *gdhB* mutants, are derepressed for a number of enzyme and uptake systems which are regulated by ammonium. This suggests that the NADP-GDH protein, but not the NAD-GDH protein, plays some role in ammonium regulation. However, NADP-GDH can only be a part of the regulatory system since deficient NADP-GDH is not an essential requirement for the absence of ammonium repression. Five other classes of mutants not repressed by ammonium, *xprD1*, *meaA*, *meaB*, *DER3* and *amrA1*, all have normal NADP-GDH.

Three main possibilities occur to us concerning the relationship between NADP-GDH and ammonium regulation. The first possibility is that NADP-GDH is a multi-functional protein which has catalytic activity and also plays a direct role in either the repression or inhibition, or both, of a number of activities. Mutation in the *gdhA* locus, the structural gene for NADP-GDH, would result in both abnormal enzyme and control activity. This seems to us at present the most likely explanation. Second, the NADP-GDH protein specified by the *gdhA* locus has only a catalytic function, but low NADP-GDH activity itself results in metabolic changes, e.g. ammonium pool size, which then result in derepression. There is evidence concerning the relationship between ammonium pool size and ammonium regulation which makes this possibility an unlikely one (J. A. Pateman and others, unpublished). The third possibility is that the *gdhA* gene specifies a product which has some fundamental but at present unknown regulatory function with respect to a number of metabolic systems. Mutations in the *gdhA* locus can simultaneously result in repression of NADP-GDH and derepression of other systems. This is an unlikely hypothesis since it requires that there are no structural gene mutants in the total of nine NADP-GDH mutants isolated. Yet structural gene mutants should be the most common class of enzyme-deficient mutants. We are currently making temperature sensitive mutants and looking at the kinetic and electrophoretic properties of the NADP-GDH from *gdhA* mutants in order to obtain more definitive evidence on this point.

If NADP-GDH is in some sense a regulatory molecule, the amino acid sequence and three-dimensional structure of the protein are obviously of great interest. Nothing is known at present of the amino acid sequence of the NADP-GDH of *Aspergillus nidulans*. However, the NADP-GDH of *Neurospora crassa* has been intensively studied by J. R. S. Fincham and co-workers (Fincham, 1962; Wootton, Chambers, Taylor & Fincham, 1973), and structural gene mutants are available which result in known amino acid replacements in the NADP-GDH protein. It is not known as yet if the NADP-GDH of *N. crassa* is involved in ammonia regulation, although preliminary work indicates that the urea uptake system in *N. crassa* may be regulated by ammonia (J. A. Pateman, unpublished). It may be that the NADP-GDH of *N. crassa* will provide the opportunity to study the effect of known amino acid replacements on the catalytic and regulatory properties of a protein.

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