The Structural Gene for NADP l-Glutamate Dehydrogenase in *Aspergillus nidulans*

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SUMMARY

A total of 41 mutants lacking NADP l-glutamate dehydrogenase (NADP-GDH) activity have been studied. All the mutations were located at the *gdhA* locus within 0-1 % recombination of *gdhA*. Two mutants, *gdhA1* and *gdhA2*, out of five examined, produced cross-reacting material which neutralized NADP-GDH antiserum. The mutant *gdhA9* has altered *Kₘ* values for all five substrates: ammonium, α-ketoglutarate, l-glutamate, NADPH and NADP. The mutant *gdhA20* had temperaturesensitive growth, abnormal ammonium-regulation characteristics and thermolabile NADP-GDH activity. These results show that *gdhA* is the structural gene for NADP-GDH.

INTRODUCTION

In *Aspergillus nidulans* a number of otherwise unrelated enzyme and transport systems are regulated by ammonium (Pateman, Kinghorn, Dunn & Forbes, 1973). These systems include nitrate reductase (Pateman & Cove, 1967), xanthine dehydrogenase (Scanzocchio & Darlington, 1968), extracellular protease (Cohen, 1972, 1973), amidases (Hynes & Pateman, 1970a, b), L-glutamate transport (Kinghorn & Pateman, 1972; Pateman, Kinghorn & Dunn, 1974), urea transport (Dunn & Pateman, 1972) and ammonium transport itself (Pateman, Dunn, Kinghorn & Forbes, 1974). Kinghorn & Pateman (1973a) described mutants *gdhA1* to *A9* which were insensitive to this control and simultaneously lacked NADP l-glutamate dehydrogenase (NADP-GDH) activity. There were two main possibilities concerning the nature of the *gdhA* mutations. They might be in a structural gene for NADP-GDH and result in altered NADP-GDH levels, bringing about the loss of ammonium control directly (Pateman *et al.* 1973), or indirectly by altering the level of an important metabolite. Alternatively, the mutations might be in a regulator gene, the altered product of which resulted in loss of ammonium control and simultaneous total repression of NADP-GDH. Since NADP-GDH might be a control element in ammonium regulation and mutation in at least one other gene, *amrBt*, can result in low NADP-GDH activity (Kinghorn & Pateman, 1973b, and unpublished observations), it was desirable to establish if *gdhA* was the structural gene for NADP-GDH. We have shown that mutation in the *gdhA* locus can result in the production of altered forms of NADP-GDH and consequently that *gdhA* is the structural gene for NADP-GDH.

METHODS

Growth of mycelium, NADP-GDH assay and genetic analysis were as described by Kinghorn & Pateman (1973a). Ammonium regulation of nitrate reductase, xanthine dehydrogenase and urea transport was determined by plate tests (Pateman *et al.* 1973).
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Media and supplements. Medium used for genetic analysis was essentially that described by Pontecorvo, Roper, Hemmons, MacDonald & Bifton (1953). Nitrogen-less minimal medium (–N medium) and carbon- and nitrogen-less minimal medium (–CN medium) were used (Kinghorn & Pateman, 1973a).

Isolation of more gdhA mutants. N-methyl-N’-nitro-N-nitrosoguanidine (NTG)-treated conidia (Adelberg, Mandel & Chen, 1965) of bi1 (a biotin auxotroph; Glasgow No. 051) were spread over –N medium + 10 mM-glutamate at 37 °C. After 2 days’ incubation the colonies were replicated on to –N medium + 100 mM-ammonium tartrate and incubated at 37 °C. Colonies unable to grow were purified and retested on –N medium + 100 mM-ammonium tartrate at 25 and 37 °C.

Preparation of antibodies against NADP-GDH. A 2 ml portion of crude extract (3 mg protein/ml) of wild-type cells, grown on –N medium + 10 mM-ammonium tartrate in 0.05 M-sodium phosphate buffer pH 7.5, was thoroughly mixed with 2 ml complete Freund’s adjuvant. This was injected intramuscularly into the large muscle of the hind leg of a New Zealand white rabbit. This procedure was repeated, with the exception that incomplete Freund’s adjuvant was used, three times at weekly intervals. The rabbit was bled before the first and after the last injection. The blood was allowed to clot by keeping overnight in a refrigerator at 4 °C. The serum was decanted, centrifuged and concentrated by dialysing overnight against saturated (NH₄)₂SO₄. The precipitate was dissolved in 0.05 M-sodium phosphate buffer pH 7.5 and dialysed against this buffer.

Cross-reacting material (c.r.m.) tests. Antiserum (0.1 ml) was added to 0.05 ml wild-type extract (1 mg protein/ml) and incubated at 37 °C for 20 min. It was found that this inhibited NADP-GDH activity by 60 to 65 %. No inhibition was observed when extracts were incubated with non-immune serum.

The ability of extracts of mutant NADP-GDH to protect wild-type NADP-GDH against anti-NADP-GDH serum was tested as follows. Antiserum (0.1 ml) was added to 0.05 ml mutant extract (1 mg protein/ml). This was incubated at 37 °C for 20 min. Then 0.05 ml of wild-type extract (1 mg protein/ml) was added and re-incubated at 37 °C for 20 min, after which the mixture was assayed for NADP-GDH activity. The c.r.m. is expressed as a percentage of test wild-type NADP-GDH activity protected by mutant extracts.

Electrophoresis. Crude cell extracts were analysed by Smithies’ (1955) starch gel technique using hydrolysed starch (Connaught Laboratories) and the discontinuous tris-buffer system of Poulik (1957). Electrophoresis was complete in about 5 h using a potential drop of 9 to 10 V/cm. On completion of electrophoresis the gels were sliced into three layers and the middle layer stained for NADP-L-glutamate dehydrogenase. Staining for NADP-GDH activity was based on the method of Markert & Moller (1959 modified by Fincham & Stadler (1965).

RESULTS

Isolation and genetics of mutants lacking NADP-GDH

In addition to ten gdhA mutants already known (Kinghorn & Pateman, 1973a; Arst & MacDonald, 1973) a further 31 mutants, gdhA11 to A42, were isolated on the basis of sensitivity to high concentrations of ammonium (–N medium + 200 mM-ammonium) at 25 °C. They were found to have decreased NADP-GDH activities, from zero to 20 % of wild type. These mutants were crossed to gdhA1 and all but three gave no wild-type recombinants in 5000 progeny. gdhA13, gdhA21 and gdhA26 showed 0.1, 0.05 and 0.05 % recombination, respectively, with gdhA1.
Table 1. Cross-reacting material in gdhA mutants

<table>
<thead>
<tr>
<th>Strain under test for NADP-GDH</th>
<th>Amount of c.r.m. (% wild-type activity protected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
</tr>
<tr>
<td>gdhA1</td>
<td>73</td>
</tr>
<tr>
<td>gdhA2</td>
<td>68</td>
</tr>
<tr>
<td>gdhA3</td>
<td>0</td>
</tr>
<tr>
<td>gdhA4</td>
<td>0</td>
</tr>
<tr>
<td>gdhA9</td>
<td>81</td>
</tr>
</tbody>
</table>

* A cell-free extract of each strain under test was incubated for 20 min at 37 °C with anti-sera against NADP-GDH. Wild-type extract was then added and re-incubated for 20 min at 37 °C. After this time, NADP-GDH activity of the mixture was assayed. Mycelium was grown at 25 °C on -N medium + 0.15 % Casamino acids + 5 mM-ammonium tartrate for 15 h before transfer to treatment media (—N medium + 5 mM-ammonium tartrate) for 5 h.

Complementation tests

We have shown that gdhA1 did not complement with the mutants gdhA2 to A9 in the heterozygous diploids; moreover, heterokaryons made between all possible combinations of gdhA1 to A9 failed to produce complementation (Kinghorn & Pateman, 1973a). Since it was not practical to construct diploids between all combinations of gdhA1 to gdhA41, complementation was tested in heterokaryons. These tests were carried out on media on which gdhA mutants grow poorly compared with the wild type: -N medium + 200 mM-ammonium at 25 °C (Kinghorn & Pateman, 1973a), and -CN medium + 1% acetate + 10 mM-ammonium at 37 °C (Arst & MacDonald, 1973). No increased growth was observed in heterokaryons between all combinations of gdhA1 to gdhA41 on either medium.

Lack of NADP-GDH activity may not be strictly correlated with high ammonium sensitivity or with growth on acetate, and this may be the reason for the lack of complementation between gdhA mutants in these tests. However, it is more likely to be due to technical reasons, since complementation has been observed in the case of NADP-GDH minus mutants in the related fungus Neurospora crassa (Pateman & Fincham, 1964; Fincham & Stadler, 1965).

Tests for cross-reacting material

Table 1 shows the ability of cell-free extracts of wild-type and gdhA1, A2, A3, A4 and A9 to protect wild-type NADP-GDH against NADP-GDH antiserum. gdhA1, gdhA2 and gdhA9 gave high levels of protection, showing that their proteins reacted with the antiserum, i.e. they were c.r.m. positive. In contrast, gdhA3 and gdhA4 gave no protection and were therefore c.r.m. negative.

Enzyme kinetics

The results presented in Table 2 show the $K_m$ values for wild-type and gdhA9 NADP-GDH for all five substrates. The $K_m$ value for each substrate was determined at saturating concentrations of the non-varying substrates. The gdhA9 enzyme had abnormally high constants for all substrates, especially ammonium and α-ketoglutarate where the mutant $K_m$ is approximately 15 times that of the wild-type. The wild-type Aspergillus nidulans $K_m$ values for ammonium, α-ketoglutarate, NADPH and NADP are similar to that of wild-type N. crassa NADP-GDH, while the $K_m$ for L-glutamate is significantly higher (Fincham & Bond, 1960).
Table 2. Properties of NADP-GDH activity in wild type and gdhA9

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>Wild type</th>
<th>gdhA9</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{NH}_4^+$</td>
<td>1.1</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-Ketoglutarate</td>
<td>3.4</td>
<td>36.4</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>0.02</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>42</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>NADP</td>
<td>0.03</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

Each value is the average of three independent experiments. See Table 1 for growth of mycelium.

Fig. 1. Starch gel electrophoresis, showing NADP-GDH activity extracts of strains of A. nidulans. For growth of mycelium, see Table 1.

Electrophoretic mobility of mutant NADP-GDH protein

From Fig. 1, depicting a starch gel stained specifically for NADP-GDH activity, it can be seen that there is only one band of activity in the wild-type strain, as was found in N. crassa (Fincham & Stadler, 1965; Stachow & Sanwal, 1967). Only the mobilities of two mutant proteins which have detectable activity are shown: gdhA8, the least detectable activity, and gdhA9, the maximum detectable activity. A faint band was observed for gdhA8 and a heavier band for gdhA9. A direct comparison of activity with the wild-type, as judged by the band density, cannot be made since the concentration of mutant protein extracts was approximately four times that of the wild-type. The bands of activity in the mutants appeared at approximately the same position as in the wild-type extracts showing that enzymes produced in the mutants gdhA9 and gdhA8 (and also the other gdhA mutants which showed NADP-GDH including the temperature-sensitive gdhA20) have similar NADP-GDH electrophoretic mobilities to the wild-type enzyme.
Table 3. Growth responses of gdhA20 at 25 and 37 °C

<table>
<thead>
<tr>
<th>Addition to -N medium</th>
<th>gdhA1</th>
<th>gdhA20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37 °C</td>
<td>25 °C</td>
</tr>
<tr>
<td>100 mM-ammonium</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>10 mM-ammonium</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>10 mM-nitrate</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>10 mM-urea</td>
<td>+</td>
<td>±</td>
</tr>
</tbody>
</table>

Growth on agar plates was scored as follows: + + , strong growth (wild-type growth with each nitrogen source and at both temperatures); + , growth; ±, poor growth; −, no growth.

Fig. 2. Temperature decay curves for NADP-GDH in crude extracts of A. nidulans. ○, Wild type; ▲, gdhA20. For growth of mycelium, see Table 1.

Table 4. Ammonium repression of nitrate reductase, xanthine dehydrogenase and thiourea transport in wild-type and gdhA20 strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Xanthine dehydrogenase*</th>
<th>Nitrate reductase*</th>
<th>Urea transport*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37 °C</td>
<td>25 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td>bit, gdhA1+</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>bit, gdhA20</td>
<td>D</td>
<td>R</td>
<td>D</td>
</tr>
<tr>
<td>Remaining gdhA mutants</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

R, system repressed by ammonium; D, system not repressed by ammonium. * Determined by plate tests.

Growth responses of gdhA mutants

All (gdhA1 to A41) the mutants except gdhA20 have growth responses similar to that of gdhA1 (Kinghorn & Pateman, 1973a). Table 3 shows the growth of gdhA20 compared with gdhA1 and wild-type with various nitrogen sources at 25 and 37 °C. The mutant gdhA20 is similar to gdhA1 in its response to inorganic nitrogen as sole nitrogen source at 37 °C, and has a wild-type response at 25 °C.
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NADP-GDH activity in gdhA20

Figure 2 shows the thermal inactivation of NADP-GDH at 60 °C in crude extracts of gdhA20. There was complete loss of enzyme activity in extracts of gdhA20 held at 60 °C for up to 3 min. There was some loss of enzyme activity when gdhA20 extracts were held at 55 °C, and no loss after treatment at 50 °C for periods up to 30 min.

Ammonium regulation

The results presented in Table 4 show that the mutation gdhA20 results in the failure of ammonium to repress nitrate reductase, xanthine dehydrogenase and urea transport at 37 °C but not at 25 °C. All other gdhA mutants isolated were derepressed for xanthine dehydrogenase, nitrate reductase and urea transport at both temperatures.

DISCUSSION

It is likely that gdhA is the structural gene for NADP-GDH. There are four main lines of evidence which indicate this. First, a total of 41 mutants have been isolated which lack normal NADP-GDH activity, and all the mutations map in the gdhA locus. Second, gdhA9 NADP-GDH shows abnormally high Michaelis constants for all five substrates, indicating the production of an abnormal enzyme protein. Third, immunologically active material found in significant amounts in three out of five gdhA mutants examined is presumably the result of missense mutations or nonsense mutations. Finally, gdhA20 produces NADP-GDH that is more sensitive to heat than the wild-type enzyme.

Although it is clear that gdhA is the structural gene for NADP-GDH, the basis of the regulatory effect is not known for certain. It remains an open question whether this is due to a defect of the NADP-GDH protein itself or to a metabolite, the concentration of which is affected by lack of NADP-GDH activity.

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REFERENCES


