A Possible Regulatory Gene for the Molybdenum-Containing Cofactor in Aspergillus nidulans

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Aspergillus nidulans has three molybdoenzymes, nitrate reductase, purine hydroxylase I and purine hydroxylase II. These three enzymes share a molybdenum-containing cofactor whose synthesis requires the integrity of five loci, designated cnxABC, cnxE, cnxF, cnxG and cnxH. Here we report the existence of a sixth locus, designated cnxJ, which might be involved in the regulation of cofactor levels. When grown in the presence, but not in the absence, of tungstate or methylammonium, strains carrying cnxJ1 or cnxJ2 have reduced molybdoenzyme levels as judged both from growth properties and enzyme determinations. A new cryosensitive cnxC- allele is also reported. Its phenotype at 37 °C (but not 25 °C) shows some similarities to that of the two cnxJ- alleles. A structural role for the cnxC (or cnxABC) product in the cofactor is tentatively suggested.

INTRODUCTION

The conversion of inorganic molybdate into molybdenum-containing moieties of many molybdoenzymes involves a number of common steps [see reviews by Hewitt & Notton (1980), Johnson (1980), Scanzocchio (1980), and Brill (1980)]. In the ascomycete fungus Aspergillus nidulans, a molybdenum-containing cofactor, whose existence was first postulated by Pateman et al. (1964), is essential to nitrate reductase and two distinct purine hydroxylases (formerly designated xanthine dehydrogenases) [reviewed by Cove (1979) and Scanzocchio (1980)]. Mutations at any of five unlinked loci, designated cnxABC, cnxE, cnxF, cnxG and cnxH, lead to loss of nitrate reductase, purine hydroxylase I and purine hydroxylase II. It is at present uncertain whether the cnxABC locus be a single gene where mutations fall into three intracistronic complementation groups or two contiguous genes, cnxA and cnxC, with cnxB- mutations being a double loss class [see discussion by Cove (1979) and Scanzocchio (1980)]. Arst et al. (1970) have presented evidence that the cnxE product is concerned with insertion of molybdenum into the cofactor. Using thermosensitive mutants, MacDonald & Cove (1974) have shown that the cnxH gene product is probably a structural component of the cofactor whereas the cnxE and cnxF products are not, indicating that they probably encode enzymes involved in cofactor biosynthesis.

Whilst studying nitrogen metabolite (i.e. ammonium) repression of the syntheses of enzymes and permeases involved in nitrogen source utilization in A. nidulans (Arst & Cove, 1969, 1973) we selected a number of mutants hypersensitive to the toxic ammonium analogue.

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methy1ammonium. Although these mutants were selected on medium containing nitrate as nitrogen source, all but one showed methy1ammonium hypersensitivity on a wide range of nitrogen sources. The remaining mutant showed interesting specificity in that it was hypersensitive only on nitrogen sources (such as nitrate) whose utilization requires catalysis by a molybdoenzyme. This mutation, plus an allelic one subsequently selected, defines an additional cofactor gene, designated cnxJ, which recombines freely with the previously identified cnx loci. There is no evidence that mutations in cnxJ can lead to loss of any or all of the three molybdoenzymes, but cnxJ− mutations effectively lower the cofactor concentration. Preliminary characterization of a cryosensitive cnxC− allele is also reported. It is consistent with a structural role for the cnxC product in the cofactor.

METHODS

Genetic techniques and strains. 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; Cove, 1979; Scagazzuchio, 1980, and references in these three articles) with the exception of cnxJ1, cnxJ2 and cnxC20 described herein.

Growth testing. Growth testing of A. nidulans has been described previously (Arst & Cove, 1969; Arst, 1981). Conidia spores of numerous independent strains were stab-inoculated on to solid media and growth was monitored after 2 to 4 d. The minimal medium described by Cove (1966) was used throughout. It contains 1% (w/v) D-glucose as carbon source. Nitrogen sources were present at final concentrations of 10 mM [nitrate, nitrite, ammonium as the (+)-tartrate], 5 mM L-proline, L-alanine, L-glutamate (monosodium salt), L-arginine (hydrochloride), glycine, y-amino-n-butyrate, urea, 735 μM (hypoxanthine) or 594 μM (uric acid). For utilization of hypoxanthine as nitrogen source via purine hydroxylase II (following Scagazzuchio et al., 1973), 5.5 μM allopurinol and 1 mM sodium nicotinate were present. Methylammonium and caesium were added as the chlorides, tungstate and molybdate as the disodium salts and chloride as the potassium salt. Unless otherwise stated, growth tests were done at 37 °C.

Selection and genetic characterization of cnxJ1, cnxJ2 and cnxC20. cnxJ1 was selected, using replica plating (Mackintosh & Pritchard, 1963), after N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis (Alderson & Hartley, 1969) of a strain of genotype pabaA1 mauA2 fwA1 (requiring p-aminobenzoate, lacking monoamine oxidase and therefore unable to catabolize methylammonium, having fawn conidial colour), because it results in inability to utilize nitrate as nitrogen source in the presence of 1 mM methy1ammonium. cnxJ2 and cnxC20 were selected, after ultraviolet mutagenesis of a strain of genotype yA2 pantoB100 (having yellow conidial colour, requiring D-pantothenate) as conferring resistance to 5 mM-chlorate on appropriately supplemented glucose-minimal medium containing 5 mM-L-proline as nitrogen source in the presence, but not in the absence, of 20 mM-tungstate. All three mutations are recessive in diploids. cnxJ1 and cnxJ2 failed to complement in a diploid and to recombine to yield any cnxJ+ progeny (able to utilize nitrate as nitrogen source in the presence of 10 mM-chlorate) out of about 2000 cross progeny tested. cnxJ1 and cnxJ2 are therefore allelic. They are also phenotypically indistinguishable. The map position of cnxJ1 is noted in Results. cnxC20 was located by haploidization (McCully & Forbes, 1965) to linkage group VIII. Eight cnx+ recombinants were recovered amongst about 70000 progeny from a cross of relevant partial genotype cnxC20 × cnxB11. In diploids cnxC20 complements cnxA5 but fails to complement cnxB11 or cnxC3.

Enzyme assays. Strains used for biochemical analysis carry pabaA1 with the exception of the wild-type strain used in some of the experiments on nitrate and nitrite reductase levels, which carries the biotin auxotrophy biA1. (In the presence of appropriate supplementation, no effect of either biA1 or pabaA1 on any enzyme involved in nitrogen metabolism has ever been detected.) For all experiments involving methy1ammonium, strains carrying, in addition, mauA2 were used. (No effect of mauA2 on enzymes other than monoamine oxidase has ever been detected.) Results are the average of at least two and usually three independent determinations. In some cases, results have been further confirmed by experiments done under slightly altered conditions for which data are not shown. Relative activities are reproducible to within 10–15% of the values shown.

For nitrate and nitrite reductase assays, mycelia were grown for 9 h at 37 °C in appropriately supplemented shaken minimal medium (Cove, 1966) containing 1% (w/v) D-glucose as carbon source and 10 mM-L-proline plus either 10 mM-nitrate or 10 mM-nitrite (as indicated) as nitrogen sources. These growth conditions are strongly inducing for both enzymes. Tungstate (disodium salt) or methy1ammonium (chloride) at the final concentrations shown was present from the time of inoculation. Extraction procedures, determination of soluble protein in extracts and assay methods for nitrate reductase (EC 1.6.6.3) and nitrite reductase (EC 1.6.6.4) have been described previously (Rand & Arst, 1977) except that nitrate reductase assays contained 10 mM (final concentration) sulphite (disodium salt) to inhibit nitrite reductase activity.
For purine hydroxylase I and urate oxidase assays, mycelia were grown for 20 h at 25 °C in appropriately supplemented shaken minimal medium (Cove, 1966) containing 1% (w/v) D-glucose as carbon source, 5 mM-urea as nitrogen source and 2-72 μM-2-thiouric acid as co-inducer. Tungstate (disodium salt) or methylammonium (chloride) at the final concentrations shown was present from the time of inoculation. Extraction procedures, determination of soluble protein in extracts and assay methods for purine hydroxylase I (EC 1.2.1.37) and urate oxidase (EC 1.7.3.3) have been described previously (Scanzocchio et al., 1973). Inhibition of purine hydroxylase I activity in vitro by tungstate cannot be followed using the standard assay because addition of tungstate to the assay mixture results in formation of a precipitate. Therefore tungstate inhibition of purine hydroxylase I in vitro was followed using NAD⁺ as the final electron acceptor. In a reaction volume of 1 ml, the assay cell contained 100 mM-orthophosphate buffer pH 7.8, 2 mM NAD⁺, 100 μM-hypoxanthine and 100 μl cell-free extract. The reaction was followed at 340 nm with a reference cell, omitting the hypoxanthine (Lewis et al., 1978).

**RESULTS**

**Growth properties of cnxJ⁻ strains**

Mutations at the cnxABC, cnxE, cnxF, cnxG and cnxH loci of *A. nidulans* have been recognized as resulting in inability to utilize the substrates of the three molybdoenzymes as nitrogen sources whilst not affecting ability to utilize the products of their reactions [reviewed by Cove (1979) and Scanzocchio (1980)]. Loss of nitrate reductase results in inability to utilize nitrate but not nitrite. Loss of purine hydroxylase I results in inability to utilize hypoxanthine but not uric acid. Loss of purine hydroxylase II results in inability to utilize nicotinate but not 6-hydroxynicotinate. If allopurinol is present to inhibit purine hydroxylase I and a low concentration of nicotinate is present to induce purine hydroxylase II, the oxidation of hypoxanthine is catalysed by purine hydroxylase II and loss of purine hydroxylase II results in inability to utilize hypoxanthine but not uric acid. *cnxJ1* and *cnxJ2* differ in phenotype from mutations at the five previously identified *cnx* loci in that they do not affect, at either 25 °C or 37 °C, utilization of nitrate, hypoxanthine, nicotinate or hypoxanthine in the presence of allopurinol and nicotinate (Table 1) unless tungstate or methylammonium is present (Table 2). Nicotinate is too poor a nitrogen source for convincing differences in inhibitor tolerance to be observed but *cnxJ⁻* strains are clearly hypersensitive when nitrate, hypoxanthine or hypoxanthine in the presence of nicotinate and allopurinol serves as nitrogen source. On a large number of other nitrogen sources tested, including nitrite and uric acid, *cnxJ⁻* strains are indistinguishable from wild-type over a range of toxic and subtoxic concentrations of tungstate and methylammonium.

Differential sensitivity of nitrate reductase levels to tungstate in *cnxJ⁻* strains can also be observed on chlorate-containing media. The basis for chlorate toxicity to growth of *A. nidulans* is controversial, but it probably involves to some extent nitrate reductase-catalysed reduction to chlorite because many mutants lacking nitrate reductase are chlorate resistant [see Cove (1976a, 1979)]. Moreover, at least some mutants which lack nitrate reductase but are nevertheless chlorate sensitive become chlorate resistant in the presence of subtoxic tungstate concentrations (Arst et al., 1979). In the presence, but not absence, of 10 to 20 mM-tungstate, *cnxJ⁻* strains, but not wild-type strains, are partially resistant to 1 to 20 mM (according to nitrogen source) chlorate on a number of nitrogen sources including L-proline (shown in Table 2), L-alanine, L-glutamate, L-arginine, glycine, γ-amino-n-butyrate, uric acid and urea.

Differential sensitivity of purine hydroxylase I levels to tungstate in *cnxJ⁻* strains can also be observed on 2-thioanithine media. 2-Thioanithine, after oxidation to 2-thiouric acid, mainly catalysed by purine hydroxylase I, prevents the conversion of yellow to green conidial pigment (Darlington & Scanzocchio, 1967; Alderson & Scanzocchio, 1967). Mutants lacking purine hydroxylase I (e.g. *cnxC3* in Table 2) are resistant to 2-thioanithine (but not to 2-thiouric acid). Tungstate partially protects *cnxJ⁻* but not wild-type strains against the effect of 2-thioanithine (Table 2).
Table 1. Utilization of molybdoenzyme substrates as nitrogen sources by wild-type and various mutant cnx strains

Growth scores: —, residual, nitrogen-starved growth; ±, weak growth; + to ++++, increasing levels of growth. Growth scores on different media are not necessarily equivalent. cnxC3 is a typical non-leaky cnx- mutation.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>NO₃⁻</th>
<th>Hypoxanthine</th>
<th>Nicotinate</th>
<th>Hypoxanthine in presence of nicotinate + allopurinol</th>
<th>NO₃⁻ in presence of 33 mM-MoO₄²⁻ in strain also carrying cnxE14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (cnxE⁺)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>cnxJ1 and J2</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>cnxC20</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>cnxC3</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

cnxE⁻ mutants can be supplemented by growth in the presence of high molybdate concentrations for restoration of molybdoenzymes as observed by growth tests, enzyme measurements, and cofactor assays (Arst et al., 1970; Ketchum & Downey, 1975). Because of the pattern of molybdate toxicity, nitrate reductase is the molybdoenzyme most readily studied for supplementation (Arst et al., 1970). High molybdate concentrations restore the ability of cnxE⁻ strains to utilize nitrate as nitrogen source (Table 1). cnx⁻ mutations abolish molybdate supplementation of cnxE⁻ lesions: unlike cnxE14 single mutants, cnxJ1 or cnxJ2 cnxE14 double mutants are unable to utilize nitrate as nitrogen source in the presence of 33 mM-molybdate (Table 1). Nevertheless, cnx⁻ mutants do not affect molybdate toxicity (Table 2), indicating that they do not affect molybdate uptake [see Arst & Cove (1970) and Arst et al. (1970)].

Can a mutation at another cnx locus have a phenotype similar to that of cnxJ⁻ mutations?

At 37 °C the growth properties of cnxC20 strains are rather similar to those of cnxJ⁻ strains (Tables 1 and 2). A clear difference does, however, emerge on media containing substrates of purine hydroxylase II: cnxC20 strains scarcely utilize nicotinate or hypoxanthine plus allopurinol and nicotinate. At 25 °C cnxC20 has a phenotype very similar to fully mutant cnxC⁻ alleles such as cnxC3 (Table 1), thus differing sharply from cnxJ1 and cnxJ2.

The basis for Cs⁺ toxicity is unclear (Arst & Page, 1973; Arst & Cove, 1973; Hynes, 1974), but it can be utilized as an in vivo probe of levels of enzymes involved in nitrogen nutrition. Resistance to Cs⁺ correlates with an increased rate of utilization of the nitrogen source, presumably leading to higher intracellular ammonium pools (as exogenous ammonium protects against Cs⁺ toxicity) (Arst & Cove, 1973; Bailey & Arst, 1975; Bailey et al., 1979; Arst et al., 1980; Arst & Bailey, 1980). Similarly, Cs⁺ hypersensitivity is associated with reduced utilization of the nitrogen source (Rand & Arst, 1977; Jones et al., 1981; Arst et al., 1981). At 37 °C, cnxC20 but not cnxJ1 or cnxJ2 leads to hypersensitivity to Cs⁺ on nitrate and hypoxanthine as nitrogen sources (Table 2). This suggests that cnxC20 reduces nitrate reductase and purine hydroxylase I levels whereas cnxJ1 and cnxJ2 do not.

Molybdoenzyme levels in cnxJ⁻ strains

The data in Table 3 show that, in agreement with its phenotype in growth tests, cnxJ1 leads to a two-fold reduction in nitrate reductase levels when growth takes place in the presence of tungstate or methylammonium but probably does not affect nitrate reductase levels in the absence of inhibitors. The specificity of the effect is confirmed by its absence when nitrite
Table 2. *In vivo* responses of wild-type and various mutant cnx strains to inhibitors under conditions involving molybdoenzyme activities or molybdenum metabolism

R, resistance to inhibitor; r, partial or intermediate resistance; S, sensitive to inhibitor; -, residual, nitrogen-starved growth because unable to utilize nitrogen source irrespective of presence of inhibitor. Hx, hypoxanthine; 2-TX, 2-thioxanthine. All tests were carried out at 37 °C. Strains carrying *mawA2*, leading to loss of monoamine oxidase (Arst & Cove, 1969; Page, 1971; Page & Cove, 1972) were used for all tests involving CH₃NH₃⁺ because the monoamine oxidase reaction both detoxifies it and allows it to be utilized as a nitrogen source. All inhibitors affect the degree of growth except 2-thioxanthine which affects the formation of green conidial pigment. Tests involving 2-thioxanthine were performed with strains which have wild-type (i.e. green) conidial colour.

Response on solid minimal medium containing the inhibitors and nitrogen sources shown

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>20 mM-WO₄²⁻</th>
<th>1 mM-CH₃NH₃⁺</th>
<th>10 mM-WO₄²⁻</th>
<th>500 μM-CH₃NH₃⁺</th>
<th>Hx in presence of nicotinate + allopurinol</th>
<th>Hx in presence of nicotinate + allopurinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (cnx+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cnxJ1 and J2</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>cnxC20</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>cnxC3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>30 mM-Cs⁺</th>
<th>10 mM-Cs⁺</th>
<th>33 mM-MoO₄²⁻</th>
<th>5 mM-CIO₃⁻</th>
<th>10 mM-WO₄²⁻</th>
<th>594 μM-2-TX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (cnx+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cnxJ1 and J2</td>
<td>r</td>
<td>r</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>cnxC20</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>r</td>
</tr>
<tr>
<td>cnxC3</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>
Table 3. **Nitrate reductase and nitrite reductase activities in wild-type and cnxJI strains under various growth conditions**

Enzyme activities are expressed as a percentage of the specific activity of a wild-type strain grown simultaneously under identical conditions except for the absence of inhibitors.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Nitrate reductase activity (%)</th>
<th>Nitrite reductase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Co-inducer NO(_3)(^-) plus:</td>
<td>Co-inducer NO(_2)(^-) plus:</td>
</tr>
<tr>
<td></td>
<td>10 mm- WO(_2)(^-)</td>
<td>20 mm- WO(_2)(^-)</td>
</tr>
<tr>
<td>Wild-type (cnx(^+))</td>
<td>100</td>
<td>38</td>
</tr>
<tr>
<td>cnxJI</td>
<td>101</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 4. **Purine hydroxylase I and urate oxidase activities in wild-type and cnxJI strains under various growth conditions**

2-Thiouric acid was present throughout as co-inducer (see Methods). Enzyme activities are expressed as a percentage of the specific activity of a wild-type strain grown simultaneously under identical conditions except for the absence of inhibitors.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Purine hydroxylase I activity (%)</th>
<th>Urate oxidase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-Thiouric acid plus:</td>
<td>2-Thiouric acid plus:</td>
</tr>
<tr>
<td></td>
<td>5 mm- WO(_2)(^-)</td>
<td>10 mm- WO(_2)(^-)</td>
</tr>
<tr>
<td>Wild-type (cnx(^+))</td>
<td>100</td>
<td>35</td>
</tr>
<tr>
<td>cnxJI</td>
<td>109</td>
<td>17</td>
</tr>
</tbody>
</table>
reductase is assayed. As both nitrate and nitrite can induce these enzymes (Pateman et al., 1967), nitrite induction was generally preferred for these experiments to avoid possible differences in the degree of induction arising from differences in the rate of nitrate reduction. Just as many mutants lacking nitrate reductase have elevated levels of nitrite reductase (Pateman et al., 1967; Cove, 1976a), the reduction of nitrate reductase levels by tungstate or methylammonium leads to elevated nitrite reductase levels except at the higher methylammonium concentration where inhibition of protein synthesis (Arst & Page, 1973) might override it.

A similar two-fold effect of cnxJ on purine hydroxylase I levels in mycelia grown in the presence of tungstate or methylammonium is seen in Table 4 where urate oxidase serves as the specificity control. The gratuitous inducer 2-thiouric acid (Scanzocchio & Darlington, 1968; Sealy-Lewis et al., 1978; Philippides & Scanzocchio, 1981) was used to avoid differential degrees of induction. It is possible that reduced induction accentuates the cnxJ- phenotype when hypoxanthine serves as nitrogen source because uric acid, the product of the purine hydroxylase I reaction, is the physiological co-inducer (Scanzocchio, 1973; Philippides & Scanzocchio, 1981). A similar accentuation of cnxJ- phenotype might apply in growth tests involving purine hydroxylase II where 6-hydroxynicotinate, the product of the reaction with nicotinate, is the physiological co-inducer (Sealy-Lewis et al., 1979).

To confirm that the cnxJ- phenotype is only revealed by growth in the presence of tungstate or methylammonium, the degrees of inhibition of nitrate reductase and purine hydroxylase I activities in cell-free extracts of a cnxJl and a wild-type strain were compared over a range of inhibitor concentrations. For both inhibitors much higher concentrations are necessary to achieve inhibition in vitro as compared to in vivo. In vitro no differences between the cnxJl and wild-type strains could be detected: 66 mM-tungstate and 100 mM-methylammonium inhibited nitrate reductase of both strains by about 50% as did 1-05 M-tungstate and 1 M-methylammonium in the case of purine hydroxylase I.

Nitrate reductase activity could not be detected in extracts of two cnxC20 strains grown at 37 °C, although full induction was evident from nitrite reductase levels (data not shown).

**Map position of cnxJ**

Haploidization (McCully & Forbes, 1965) located cnxJ to linkage group VII. This linkage group contains two other genes whose integrity is necessary to one or more molybdenoenzymes, the cofactor gene cnxF and hxB, a gene whose product is probably involved in a post-transcriptional modification essential to purine hydroxylases I and II (Scanzocchio, 1980). However, mapping crosses showed free recombination between cnxJ and both cnxF8 and hxB13. Further crosses located cnxJ (designated mahA10 in earlier publications) about 4 centimorgans from the prn gene cluster involved in L-proline catabolism but on the side opposite to that of the sF, pantOB and nimO genes (Arst & MacDonald, 1978). This position makes it useful as a flanking marker in fine-structure mapping of the prn cluster (Arst & MacDonald, 1978; Arst et al., 1981).

**DISCUSSION**

*Designation of cnxJ as a gene affecting the molybdenum-containing cofactor.* The fact that cnxJ- mutations only affect levels of the three molybdenoenzymes strongly suggests that they affect the molybdenum-containing cofactor and thus define a sixth cnx locus in *A. nidulans*. The nature of the two inhibitors which reveal the cnxJ- phenotype is entirely consistent with this proposal. Tungstate, a molybdate analogue, is probably able to lower the concentration of active molybdenum-containing cofactor by competition with molybdate for uptake into the cell and, once inside, for incorporation into the cofactor (Arst, 1968; Arst & Cove, 1970;
Arst et al., 1970; Sorger et al., 1974; Dantzig et al., 1978). Synthesis of the molybdenum-containing cofactor is apparently strongly reduced when wild-type *A. nidulans* is grown in the presence of ammonium (Garrett & Cove, 1976) and it is quite possible that methylammonium can mimic this effect (Arst & Cove, 1969, 1973; Arst & Page, 1973; Bartnik et al., 1973; Hynes, 1974). Thus, although tungstate and methylammonium probably have almost nothing in common structurally or metabolically, they might share the ability, albeit through different means, to reduce molybdenum-containing cofactor concentrations.

This cnx designation derives further support from the fact that a leaky mutation, *cnxC20*, in one of the five previously established *cnx* loci has a phenotype at 37 °C which is somewhat similar to that of the two *cnxJ*− mutations. The fact that the *cnxC20* phenotype also differs strikingly in some respects from that of *cnxJ1* and *cnxJ2* is hardly surprising in view of the likelihood that the two gene products concerned play extremely different roles (vide infra).

**Role of cnxJ.** In assessing the role of the *cnxJ* gene, the key question is whether *cnxJ1* and *cnxJ2* result in complete loss of *cnxJ* function or whether a deletion of the *cnxJ* gene would have a more extreme phenotype, such as that of mutations at the other five *cnx* loci. At present the only evidence is negative: despite the characterization of very large numbers of mutations leading to loss of nitrate reductase, purine hydroxylase 1 and purine hydroxylase II, spontaneous or induced by a variety of mutagens and selected using a number of different techniques (Pateman et al., 1964; Alderson & Sczzocchio, 1967; Alderson & Hartley, 1969; Alderson & Scott, 1971; Scott & Alderson, 1971: Cove, 1976b, 1979; Scott et al., 1977; Sczzocchio, 1980), no *cnxJ*− mutation leading to lack of utilization of substrates of any or all of the three molybdoenzymes has even been obtained. Unless the *cnxJ* product has some additional and essential role(s), it therefore seems extremely unlikely that mutations in *cnxJ* can lead to loss of the molybdoenzymes. If the *cnxJ* product be dispensable for cofactor synthesis, it is almost certainly not a structural component of the cofactor. It could only be an enzyme involved in cofactor synthesis if it catalyses a reaction which proceeds spontaneously at an appreciable rate or which is catalysed by an alternative enzyme or which enhances the affinity of the cofactor for the three apoenzymes without significantly affecting holoenzyme efficiencies. A more attractive hypothesis, formally equivalent to the third of these possibilities on present evidence, is that *cnxJ1* and *cnxJ2* lead to a reduction in cofactor concentration. On this model *cnxJ* might be a positive-acting regulatory gene whose product is necessary for a high, but not a moderate, level of cofactor synthesis. [Under most growth conditions, the cofactor is probably present in excess (Johnson, 1980).] It would not, however, be easy to propose a physiological role for such a regulatory gene. Cofactor synthesis seems not to be regulated by the positive-acting regulatory genes controlling nitrate reductase or purine hydroxylase I synthesis (Sczzocchio & Darlington, 1967; Garrett & Cove, 1976). Garrett & Cove (1976) found no evidence for induction of cofactor synthesis by co-inducers of the three molybdoenzymes. In their experiments the only addition to the growth medium which influenced cofactor levels was ammonium, which apparently represses synthesis of a gene product(s) necessary for cofactor synthesis and/or inhibits an enzyme(s) involved in cofactor synthesis. The ability of the ammonium analogue methylammonium to reveal the *cnxJ*− phenotype would seem to argue against mediation of the ammonium effect by the *cnxJ* product.

**Role of cnxC.** As *cnxC20* strains utilize nitrate well at 37 °C, it is possible that nitrate reductase from *cnxC20* strains is too labile to withstand extraction. This might suggest that the gene product concerned be a structural component of the cofactor. Two other examples where it has not been possible to extract enzymes from cryosensitive mutants of *A. nidulans* grown at a permissive temperature are known. A cryosensitive mutation in the structural gene for proline oxidase prevents detection of that activity in vitro (Jones et al., 1981). And a cryosensitive mutation in *hxA*, the structural gene (Sczzocchio & Sealy-Lewis, 1978; Sczzocchio, 1980) for the apoenzyme of purine hydroxylase I, has similarly thwarted all
A structural involvement for the \textit{cnxC} (or \textit{cnxABC}) product would be easily reconciled with the fact that the phenotype of \textit{cnxC20} is conditional (i.e. cryosensitive) with respect to nitrate reductase and purine hydroxylase I but virtually non-conditional with respect to purine hydroxylase II [although the requirement of purine hydroxylase II for the cofactor might simply be more stringent — see Scazzocchio (1980)]. On present evidence, however, this conclusion should be regarded as strictly tentative. The cofactor is likely to have a rather low molecular weight [see discussion by Johnson (1980) and Scazzocchio (1980)]. It is therefore difficult to see how it could include a polypeptide component in addition to that specified by \textit{cnxH}.

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Aspergillus Mo cofactor regulatory gene


