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 & Notten (1980), Johnson (1980), Scazzocchio (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 Scazzocchio (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 Scazzocchio (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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methylammonium. Although these mutants were selected on medium containing nitrate as nitrogen source, all but one showed methylammonium 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; Scazzocchio, 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). Conidiospores 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, nicotinate and 6-hydroxynicotinate as the sodium salts; ammonium as the (+)-tartrate], 5 mM [L-proline, L-alanine, L-glutamate (monosodium salt), L-arginine (hydrochloride), glycine, γ -amino-*n*-butyrate, urea], 735 μ M (hypoxanthine) or 594 μ M (uric acid). For utilization of hypoxanthine as nitrogen source via purine hydroxylase II (following Scazzocchio *et al.*, 1973), 5.5 μ M-allopurinol and 1 mM-sodium nicotinate were present. Methylammonium and caesium were added as the chlorides, tungstate and molybdate as the disodium salts and chlorate 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-methylammonium. *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-tungstate) out of about 2000 cross progeny tested. *cnxJ1* and *cnxJ2* are therefore allelic. They are also phenotypically indistinguishable. The map position of *cnxJ* is noted in Results. *cnxC20* was located by haploidization (McCully & Forbes, 1965) to linkage group VIII. Eight *cnx*⁺ recombinants were recovered amongst about 70 000 progeny from a cross of relevant partial genotype *cnxC20* $\times$ *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 methylammonium, 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 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 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 (Scazzocchio *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 Scazzocchio (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 (1976*a*, 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-thioxanthine media. 2-Thioxanthine, after oxidation to 2-thiouric acid, mainly catalysed by purine hydroxylase I, prevents the conversion of yellow to green conidial pigment (Darlington & Scazzocchio, 1967; Alderson & Scazzocchio, 1967). Mutants lacking purine hydroxylase I (e.g. *cnxC3* in Table 2) are resistant to 2-thioxanthine (but not to 2-thiouric acid). Tungstate partially protects *cnxJ⁻* but not wild-type strains against the effect of 2-thioxanthine (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; $\pm$, weak growth; + to +++, increasing levels of growth. Growth scores on different media are not necessarily equivalent. *cnxC3* is a typical non-leaky *cnx*[−] mutation.

Relevant genotype	Growth with nitrogen source and temperature shown								
	NO ₃ [−]		Hypoxanthine		Nicotinate		Hypoxanthine in presence of nicotinate + allopurinol		NO ₃ [−] in presence of 33 mM-MoO ₄ ^{2−} in strain also carrying <i>cnxE14</i> 37 °C
	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	
Wild-type (<i>cnx</i> ⁺)	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>cnxJ1</i> and <i>J2</i>	+++	+++	+++	+++	+++	+++	+++	+++	−
<i>cnxC20</i>	−	+++	−	++	−	+	−	±	−
<i>cnxC3</i>	−	−	−	−	−	−	−	−	−

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). *cnxJ*[−] 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, *cnxJ*[−] 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 *mauA2*, leading to loss of monoamine oxidase (Arst & Cove, 1969; Page, 1971; Page & Cove, 1972) were used for all tests involving CH_3NH_2^+ 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.

Relevant genotype	Response on solid minimal medium containing the inhibitors and nitrogen sources shown										
	20 mM-WO ₄ ²⁻ + NO ₃ ⁻	1 mM-CH ₃ NH ₃ ⁺ + NO ₃ ⁻	10 mM-WO ₄ ²⁻ + Hx	500 μM-CH ₃ NH ₃ ⁺ + Hx	200 μM-WO ₄ ²⁻ + Hx in presence of nicotinate + allopurinol	50 μM-CH ₃ NH ₃ ⁺ + Hx in presence of nicotinate + allopurinol	30 mM-Cs ⁺ + NO ₃ ⁻	10 mM-Cs ⁺ + Hx	33 mM-MoO ₄ ²⁻ + NH ₄ ⁺	5 mM-ClO ₃ ⁻ + 10 mM-WO ₄ ²⁻ + L-proline	594 μM-2-TX + 10 mM-WO ₄ ²⁻ + L-proline
Wild-type (cnx ⁺)	R	R	R	R	R	R	r	r	S	S	S
cnxJ1 and J2	S	S	S	S	S	S	r	r	S	S	r
cnxC20	S	S	S	S	S/-	S/-	S	S	S	R	R
cnxC3	-	-	-	-	-	-	-	-	-	-	-
Wild-type (cnx ⁺)	r	r	r	S	S	S	r	r	S	S	r
cnxJ1 and J2	r	r	r	S	S	S	r	r	S	S	r
cnxC20	S	S	S	R	R	R	S	S	R	R	R
cnxC3	-	-	-	R	R	R	-	-	-	-	-

Table 3. Nitrate reductase and nitrite reductase activities in wild-type and *cnxJ1* 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.

Relevant genotype	Nitrate reductase activity (%)										Nitrite reductase activity (%)			
	Co-inducer NO ₃ ⁻ plus:					Co-inducer NO ₃ ⁻ plus:					Co-inducer NO ₂ ⁻ plus:			
	Nothing	10 mM- WO ₄ ²⁻	20 mM- WO ₄ ²⁻	10 mM- WO ₄ ²⁻	1 mM- CH ₃ NH ₃ ⁺	Nothing	10 mM- WO ₄ ²⁻	20 mM- WO ₄ ²⁻	1 mM- CH ₃ NH ₃ ⁺	2.5 mM- CH ₃ NH ₃ ⁺	Nothing	10 mM- WO ₄ ²⁻	20 mM- WO ₄ ²⁻	2.5 mM- CH ₃ NH ₃ ⁺
Wild-type (<i>cnxJ</i> ⁺)	100	38	26	100	21	20	55	46	29	84	100	174	160	142
<i>cnxJ1</i>	101	22	13	75	10	10	34	29		82	95	162	174	138

Table 4. Purine hydroxylase I and urate oxidase activities in wild-type and *cnxJ1* 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.

Relevant genotype	Purine hydroxylase I activity (%)										Urate oxidase activity (%)			
	Co-inducer NO ₃ ⁻ plus:					Co-inducer NO ₃ ⁻ plus:					Co-inducer NO ₂ ⁻ plus:			
	Nothing	5 mM- WO ₄ ²⁻	10 mM- WO ₄ ²⁻	20 mM- WO ₄ ²⁻	50 μM- CH ₃ NH ₃ ⁺	Nothing	5 mM- WO ₄ ²⁻	10 mM- WO ₄ ²⁻	20 mM- WO ₄ ²⁻	50 μM- CH ₃ NH ₃ ⁺	Nothing	20 mM- WO ₄ ²⁻	50 μM- CH ₃ NH ₃ ⁺	
Wild-type (<i>cnxJ</i> ⁺)	100	35	15	6	116	100	35	15	6	116	100	52	92	
<i>cnxJ1</i>	109	17	8	2	67	109	17	8	2	67	86	58	80	

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, 1976*a*), 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 *cnxJ1* 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 (Scazzocchio & Darlington, 1968; Sealy-Lewis *et al.*, 1978; Philippides & Scazzocchio, 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 (Scazzocchio, 1973; Philippides & Scazzocchio, 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 *cnxJ1* 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 *cnxJ1* 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 *cnxJ1* to linkage group VII. This linkage group contains two other genes whose integrity is necessary to one or more molybdoenzymes, 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 (Scazzocchio, 1980). However, mapping crosses showed free recombination between *cnxJ1* and both *cnxF8* and *hxB13*. Further crosses located *cnxJ1* (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 molybdoenzymes 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 I 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 & Scazzocchio, 1967; Alderson & Hartley, 1969; Alderson & Scott, 1971; Scott & Alderson, 1971; Cove, 1976*b*, 1979; Scott *et al.*, 1977; Scazzocchio, 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 (Scazzocchio & 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 (Scazzocchio & Sealy-Lewis, 1978; Scazzocchio, 1980) for the apoenzyme of purine hydroxylase I, has similarly thwarted all

attempts at extraction of that enzyme [Lycan, Sealy-Lewis & Scazzocchio as cited in Scazzocchio (1980)].

A structural involvement for the *cnxC* (or *cnxABC*) product would be easily reconciled with the fact that the phenotype of *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 *cnxH*.

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