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Autoregulation of the Synthesis of Nitrate Reductase in *Aspergillus nidulans*

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In *Aspergillus nidulans*, the syntheses of nitrate and nitrite reductases are induced by nitrate, and are repressed by ammonium. It is possible in wild-type strains to overcome partially the repressive effect of ammonium, by the addition of high concentrations of nitrate to the growth medium. Mutations which lead to the production of abnormal nitrate reductase affect in addition the control of the synthesis of the nitrate-metabolizing enzymes, which in these strains are produced constitutively. That this is not due to the accumulation of an internal inducer has now been shown, as these mutants have been found to be unable to respond to nitrate induction in the presence of ammonium in the same way as do wild-type strains. To explain these findings, we propose that the nitrate reductase molecule provides the recognition site for nitrate in the control system, such that when it is not complexed with nitrate it acts as a co-repressor, and, when it is complexed, as a co-inducer.

The synthesis of nitrate, nitrite, and hydroxylamine reductases are subject to induction by nitrate or nitrite (4, 8), and to repression by ammonium (1, 8). The repressive effect of ammonium is more powerful than the inductive effect of nitrate; when these two ions are added in equal concentrations, enzyme synthesis is largely inhibited (1). Data will be presented in this paper which indicate that the synthesis of these enzymes can still to some extent be induced by nitrate, even though the fungus is growing in the presence of ammonium.

It has been reported elsewhere (7, 8) that the majority of mutant strains of *Aspergillus* which lack nitrate reductase activity, and which are therefore thought to produce an abnormal nitrate reductase, synthesize nitrite and hydroxylamine reductase constitutively, although this synthesis is still subject to repression by ammonium. The same is also true of cytochrome *c* reductase, which is another nitrate-induced enzyme activity and which has been shown to be an alternative activity of the nitrate reductase protein (3, 7); this indicates that these mutant strains also synthesize the abnormal nitrate reductase constitutively.

The behavior of these mutants is open to interpretation by two hypotheses (2, 8). First, the constitutive synthesis of the nitrate-induced enzymes by strains lacking functional nitrate reductase might result from the accumulation intracellularly of nitrate, as a result of the absence of nitrate reductase activity. This hypothesis is supported by the finding that not all strains lacking nitrate reductase synthesize the enzymes constitutively, and that in general some nitrate reductase activity can be detected in the strains which are inducible. However, Pateman et al. (8) recently presented data which are not compatible with this hypothesis, and Cove (2) described various characteristics of the induction kinetics of nitrate reductase and of cytochrome *c* reductase in wild-type and in mutant strains which appear to be open to interpretation by a similar hypothesis but which in the final analysis prove to be inconsistent with it.

The alternative hypothesis proposes that nitrate reductase plays an intimate role in the regulation both of its own synthesis and of the synthesis of nitrite and hydroxylamine reductase. Mutants producing abnormal nitrate reductase would therefore show both abnormal nitrate reductase activity and abnormalities in the control of these enzymes.

Data will be presented in this paper which are incompatible with the first hypothesis, and which show that these mutant strains are abnormal in another control function, thus making the second hypothesis still more attractive. A model of the possible regulatory role of nitrate reductase will be constructed in the light of these findings and

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of other facts known about the regulation of these enzymes.

MATERIALS AND METHODS

The origin of the strains (4, 7, 8), the composition of the media (1), the techniques used for obtaining mycelium (1) and for extracting and assaying enzymes (1, 8), with the exception described below, have been given elsewhere.

Extraction of mycelium. Mycelium was extracted in 10 times its weight of 0.1 M Na_2HPO_4 solution to which was added L-cysteine (10^{-2} M). Extraction was effected by ultrasonic disintegration (Dawe Instruments Ltd. Soniprobe type 1130/1, with a type 1A convertor). To minimize overheating, the extraction was performed in a thin glass tube, immersed in freezing mixture at −10 C. Four extraction periods of 30 sec were used, with a 30-sec interval between each.

RESULTS

A total of 3 prototrophic strains, 12 *cnx* mutants, which are thought to lack nitrate reductase activity because of their inability to synthesize a molybdenum cofactor essential for that activity (5, 7), and 13 *niaD* mutants, which are thought to carry mutations in the nitrate reductase structural gene (7), were used. Table 1 summarizes the growth and enzyme characteristics of the strains used. All the strains carrying *cnx* and *niaD* mutations were of the type which synthesize nitrite reductase constitutively. The strains were inoculated into liquid minimal medium and grown by shaken culture. The treatments are described fully in Table 2, and the levels of nitrite reductase specific activities found with these treatments are given in Fig. 1. The nitrate reductase specific activities for the prototrophic strains found with these treatments are given in Fig. 2. In no case was a nitrate reductase specific activity above 2 found in a strain carrying a *cnx* or a *niaD* mutation. The levels of hydroxylamine reductase found in all strains were in general proportional to the

TABLE 1. *Summary of growth and enzyme characteristics of strains used*

Strain	Ability to use as a nitrogen source				Enzyme characteristics		
	NO_3^-	NO_2^-	NH_4^+	Hypoxanthine	Nitrate reductase	Nitrite and hydroxylamine reductase	Xanthine dehydrogenase
Prototroph	+	+	+	+	Synthesis induced by nitrate	Synthesis induced by nitrate	Synthesis induced by uric acid
cnx (*38 of* 45)	−	+	+	−	None detectable	Synthesis constitutive	None detectable
(7 of 45)	−	+	+	−	Trace detectable	Synthesis induced by nitrate	None detectable
nia D (17 of 20)	−	+	+	+	None detectable	Synthesis constitutive	Synthesis induced by uric acid
(3 of 20)	−	+	+	+	Trace detectable	Synthesis induced by nitrate	Synthesis induced by uric acid

TABLE 2. *Summary of treatments used*[a]

Description or source	Treatment 1	Treatment 2	Treatment 3
Initial nitrogen source	10^{-2} M ammonium	As in treatment 1	As in treatment 1
15 hr after inoculation	10^{-2} moles of ammonium added per liter of medium	As in treatment 1	As in treatment 1
17 hr after inoculation	Harvested	10^{-2} moles of ammonium + 10^{-2} moles of nitrate added per liter of medium	10^{-2} moles of ammonium + 10^{-1} moles of nitrate added per liter of medium
20 hr after inoculation		Harvested	Harvested
NO_3^- to NH_4^+ ratio	0	1/3	10/3

[a] Ammonium added as ammonium tartrate; nitrate added as $NaNO_3$.

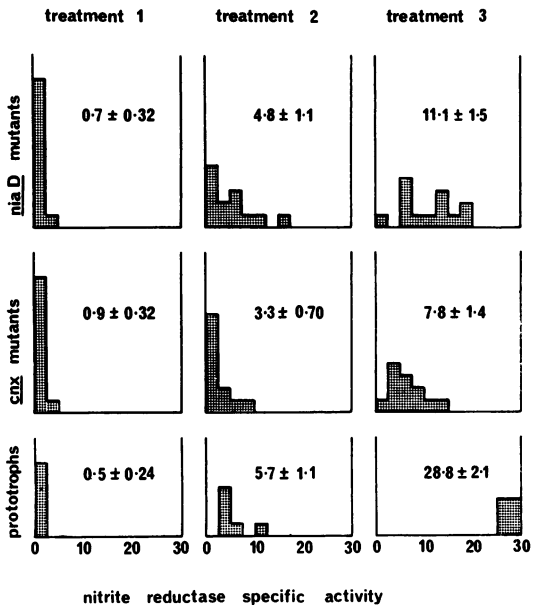

FIG. 1. *Nitrite reductase specific activities of mutant and prototrophic strains grown in the presence of various concentrations of nitrate and ammonium. Details of the three treatments used are given in Table 2. Each histogram shows the distribution of nitrite reductase specific activities found for that treatment, in the various strains investigated. The figures above each histogram give the mean specific activity for that treatment, and its standard error.*

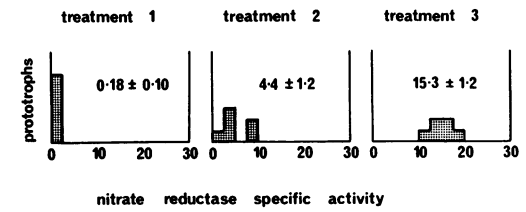

FIG. 2. *Nitrate reductase specific activities of prototrophic strains grown in the presence of various concentrations of nitrate and ammonium. Details as in Fig. 1.*

levels of nitrite reductase, and so these data are not presented.

DISCUSSION

The nitrite and nitrate reductase activities in prototrophic strains were higher for treatment 2 than for treatment 1 (Fig. 1 and 2). Thus, even in the presence of an excess of ammonium, nitrate induced some synthesis of these enzymes. When an excess of nitrate was added (treatment 3), the activities of these enzymes were 50 to 60 times greater than the activities in mycelium growing on ammonium alone. Although it was impossible to observe the effects of these treatments on nitrate reductase in strains carrying either *cnx* or *niaD* mutations, nitrate was far less effective in competing with ammonium to cause induction of nitrite reductase (Fig. 1).

As mentioned in the introduction, these mutants synthesize nitrite reductase constitutively in the absence of ammonium. Two possible explanations of this latter phenomenon were given there. The first proposed that these strains accumulated nitrate intracellularly as a result of their lack of nitrate reductase activity. According to this hypothesis, these mutant strains should respond to nitrate in the presence of ammonium as much or more than do prototrophic strains; it would be predicted that the nitrate concentration in the cells of these strains as a result of treatments 2 and 3 would be as high or higher than that in prototrophic strains. The fact that the mutants respond far less therefore contradicts this first hypothesis.

The production of an abnormal nitrate reductase thus simultaneously has three effects: (i) loss of nitrate reductase catalytic activity; (ii) constitutive synthesis of nitrite and hydroxylamine reductases, providing ammonium is not present; (iii) partial loss of response to nitrate induction in the presence of ammonium. The most likely explanation of these phenomena is that nitrate reductase has two functions in the cell, one catalytic and the other regulatory. Alterations to nitrate reductase as a result of mutation either in its structural gene or in genes concerned with the synthesis of a cofactor would then result in altered catalytic activity and in abnormalities in regulation.

Pateman and Cove (6) put forward a model to account for the characteristics of mutations in the *nir* gene, a gene distinct from both the nitrate and nitrite reductase structural genes and from the genes responsible for the synthesis of the molybdenum cofactor. They proposed that *nir* is a regulator gene, whose product must be necessary both to allow and to prevent expression of the nitrate and nitrite reductase structural genes. The immediate product of *nir* is assumed to be a repressor substance, which prevents enzyme synthesis, but which in the presence of nitrate is converted to an inducer substance required for enzyme synthesis.

To reconcile this model with the fact that nitrate reductase is also involved in this regulation system, we propose that the immediate product of the regulator gene *nir* is not a repressor substance which is converted to an inducer substance by nitrate, but instead is an inducer substance which is converted to a repressor substance by the

nitrate reductase protein. However, the nitrate reductase protein is unable to effect this conversion when it complexes with nitrate, which it must do as part of its catalytic function. Thus, nitrate reductase is assumed to behave to some extent like a co-repressor. Mutants unable to make normal nitrate reductase would be constitutive as a result of all their regulator substance being in the form of inducer. The model is also consistent with the minority class of mutants, which, while producing subnormal levels of nitrate reductase, retain inducible synthesis. Here it is assumed that the requirement of functional nitrate reductase to effect conversion of the inducer to repressor is far lower than the requirement to utilize nitrate as a nitrogen source. It has been mentioned above that some functional nitrate reductase can be detected in all of the inducible strains.

Thus modified, the model still does not explain the differences between mutant and prototrophic strains with respect to their response to induction by nitrate in the presence of ammonium. To account for this, we propose that the model be further modified as follows: that the conversion of the inducer to repressor can be brought about by ammonium, as well as by nitrate reductase when it is not complexed with nitrate. However, in this case nitrate reductase, when it is complexed with nitrate, can to some extent prevent ammonium from effecting this conversion. The final model is given in Fig. 3.

This model accounts for the abnormalities shown by the *nir*^{c} allele with respect to ammonium repression reported by Pateman and Cove (6), and would also allow regulation to be affected in two ways in strains producing abnormal nitrate reductase. In the absence of ammonium, the enzymes would be synthesized constitutively, as the abnormal nitrate reductase would not convert the regulator substance from its inducer to its repressor form. In the presence of ammonium, synthesis of the enzymes would be repressed because ammonium would then bring about this conver-

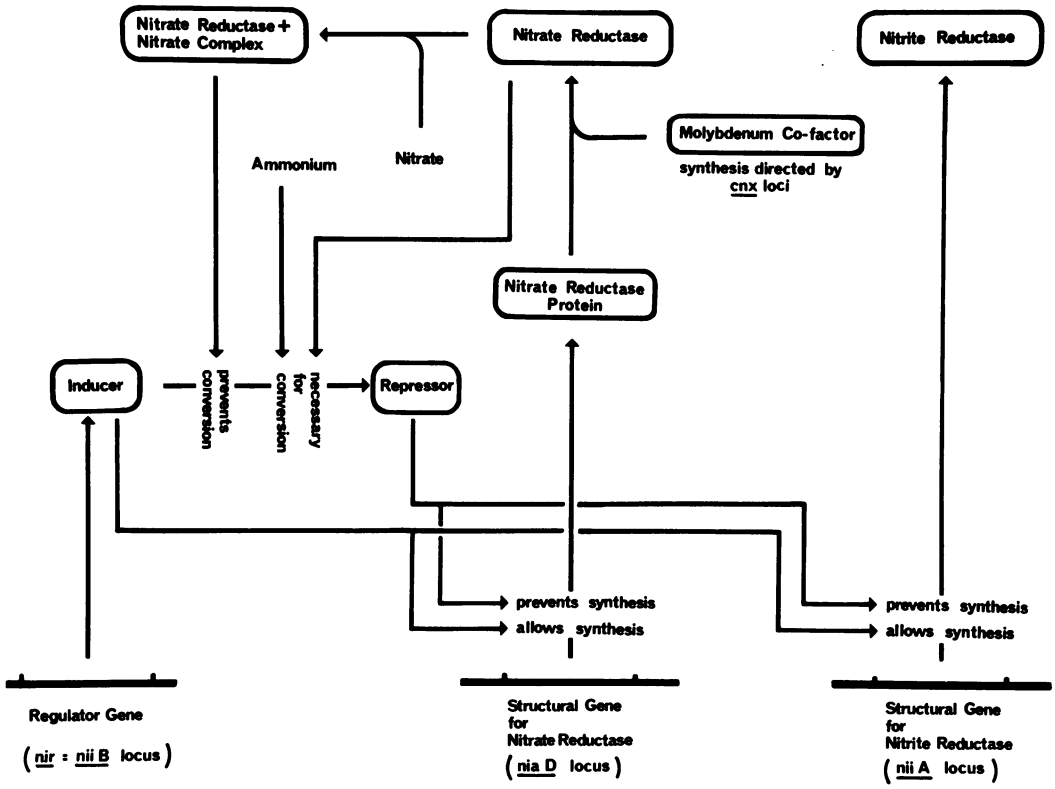

FIG. 3. *Model illustrating the possible mechanisms of the control of nitrate and nitrite reductase production in Aspergillus nidulans. The nir gene produces a substance which is necessary for expression of the structural genes. This substance is converted to a repressor, which prevents the expression of the structural genes, either by ammonium or by nitrate reductase when it is not complexed with nitrate. Complexed with nitrate, nitrate reductase interferes with the conversion of the substance to a repressor.*

sion. However, the interaction of nitrate with ammonium would also be abnormal in these strains, because nitrate reductase mediates this interaction.

LITERATURE CITED

1. Cove, D. J. 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulanus*. Biochim. Biophys. Acta **113**:51–56.
2. Cove, D. J. 1967. Kinetic studies of the induction of nitrate reductase and cytochrome *c* reductase in the fungus *Aspergillus nidulanus*. Biochem. J. **104**:1033–1039.
3. Cove, D. J., and A. Coddington. 1965. Purification of nitrate reductase and cytochrome *c* reductase from *Aspergillus nidulanus*. Biochim. Biophys. Acta **110**:312–318.
4. Cove, D. J., and J. A. Pateman. 1963. Independently segregating loci concerned with nitrate reductase activity in *Aspergillus nidulanus*. Nature **198**:262–263.
5. Cove, D. J., J. A. Pateman, and B. M. Rever. 1964. Genetic control of nitrate reduction in *Aspergillus nidulanus*. Heredity **19**:529.
6. Pateman, J. A., and D. J. Cove. 1967. Regulation of nitrate reduction in *Aspergillus nidulanus*. Nature **215**:1234–1237.
7. Pateman, J. A., D. J. Cove, B. M. Rever, and D. B. Roberts. 1964. A common co-factor for nitrate reductase and xanthine dehydrogenase which also regulates the synthesis of nitrate reductase. Nature **201**:58–60.
8. Pateman, J. A., B. M. Rever, and D. J. Cove. 1967. Genetic and biochemical studies of nitrate reductase in *Aspergillus nidulanus*. Biochem. J. **104**:103–111.