The Role of Molybdenum in Formation of the NADPH-nitrate reductase by Aspergillus nidulans

R. J. Downey

Department of Zoology and Microbiology

Ohio University

Athens, Ohio 45701

Received December 15, 1972

ABSTRACT

Non-nitrate reducing mutants of Aspergillus nidulans have been noted to produce either a nitrate inducible or constitutive NADPH-cytochrome c reductase which resides in either a 4.5s or a 7.8s protein. The latter closely resembles the nitrate inducible, FAD dependent NADPH-nitrate reductase from the wild type. Measurement of flavin adenine dinucleotide (FAD) and molybdenum (Mo) in these two proteins revealed significant differences particularly in Mo. The concepts that a nitrate inducible nia gene product constitutes the major flavin bearing component of the enzyme and that a constitutively produced cnx gene product is implicated in formation of the larger Mo bearing multimer are further supported.

INTRODUCTION

Several lines of evidence attest to the multimeric nature of the nitrate inducible NADPH-nitrate reductase in fungi (1,2,3,4) and higher plants (5). A universal feature of the molybdoflavoprotein from wild type strains is the ability to reduce cytochrome c as well as nitrate. In Aspergillus nidulans, mutants lacking nitrate reductase activity synthesize one or more components of the native enzyme following induction by nitrate. The molybdoprotein com-

ponent of the enzyme multimer is presumed lacking or faulty in cofactor (cnx) mutants on the basis of biochemical and genetic evidence (6,7,8).

Recently an investigation of a variety of mutants of Aspergillus which bear alterations in either the structural gene (niaD) or cofactor genes (cnx) for nitrate reductase and which produce both constitutive and inducible NADPH-cytochrome c reductase revealed this activity resided in either a 4.5s or 7.8s protein (3,4). The above enzyme activity was observed to be associated with one or the other of these two proteins in all but two strains among the 26 examined. Subsequently, quantitative analyses of FAD and Mo in purified mutant enzyme were conducted in an effort to detect differences, if any, in the level of these essential cofactors in the light (4.5s) and normal (7.8s) mutant enzyme.

MATERIALS AND METHODS

The wild type (bi-1) and mutant strains used in this investigation were a gift of Dr. D.J. Cove, Department of Genetics, University of Cambridge, Cambridge, England. The mutants were tested for inability to grow on solid minimal medium (8,9) containing nitrate (10mM) or hypoxanthine (10mM) as sole nitrogen source. The NADPH-nitrate reductase and NADPH-cytochrome c reductase from both wild type and mutant strains was purified and sedimentation coefficients were determined by methods previously described (3,4).

Quantitative analyses of flavin as FAD were determined by the methods of Burch et al (14) and DeLuca et al (12) respectively. Flavin as FMN was measured by the technique of Huennekens and Felton (13). Molybdenum was determined by the procedure of Johnson and Arkley (16). Protein was determined by the Folin-phenol method (10).

RESULTS

Analysis of bound flavin by two different methods revealed that the FAD levels in purified enzyme from mutants which synthesized a 4.5s or a 7.8s nitrate inducible NADPH-cytochrome c reductase were comparable to the level in

TABLE 1

Flavin analysis of NADPH-nitrate reductase from wild type and mutant strains of Aspergillus nidulans

Method of estimation		Flavin* nmoles per mg protein				
	Strain →	bi-1	cnxB-11	cnxF-9	niaD-17	niaD-8
	(s value) →	7.8	4.5	7.8	4.5	7.8
I	Total flavin by reduction with hydrosulfite**	5.1	4.9	4.8	4.5	4.7
II	FAD by D-amino acid oxidase-lactic dehydrogenase	6.4	7.3	5.2	4.9	4.8
III	FMN by NADPH-cytochrome c reductase	< 0.6	< 0.4	< 0.7	< 0.5	< 0.7

* Calculated on the basis that all of the flavin was recovered after heat and acid treatment. < = value rounded to next largest significant figure.

** $\Delta A_{450} = 11.3 \times 10^3 \text{ cm}^2 \text{ M}^{-1}$ (difference between oxidized and reduced).

the purified wild type enzyme. (Table 1) This would suggest that the light (4.5s) protein is the FAD bearing component. Characteristic low levels of FMN were noted in purified enzyme from these mutants. Since purification procedures create a flavin dependence in the NADPH-nitrate reductase of NADPH-cytochrome c reductase, it is apparent that the FAD cofactor bears both a tight and a loose association with the apoenzyme.

The observation by Cove et al (7,11) that 33 mM molybdate partially restores growth of cnxE mutants on nitrate coupled to the fact that no cnx mutations have been noted to confer molybdate resistance under any conditions make it unlikely that their phenotype stems from defective molybdate transport. In order to gather more direct evidence, analysis of total Mo in mycelium from wild type and representative cnx mutants was conducted after

TABLE 2

Mycelial molybdenum levels in wild type and mutant strains of *Aspergillus nidulans*

Strain	Mo* (μg/gm dry wt.)
bi-1	5.30 (±0.42)
cnxB-11	9.66 (±0.37)
cnxG-4	10.12 (±0.29)
niaD-10	15.77 (±0.61)
niaD-17	16.38 (±0.17)

* Values are the mean of four separate measurements on three different batches of mycelium (15). The mean deviation is shown in parentheses.

growth on a minimal medium containing 10 mM urea and 10 mM sodium nitrate.

The Mo levels in mycelium from the two *cnx* mutants examined were about twice that in the wild type. (Table 2) The *niaD* mutants exhibited a three-fold higher level. The inability of *niaD* and *cnx* mutants to form a functional NADPH-nitrate reductase thus does not appear to be due to retarded transport or inordinately low levels of intracellular Mo. The actual quantity of Mo in the mutant nitrate reductase complex, however, may be significantly less than in the native enzyme. If so, this fact would be the first of several needed to attribute nitrate non-reactivity to an outright Mo deficiency in the nitrate reductase multimer.

To support this notion, an analysis of Mo was conducted on the enzyme isolated from wild type and mutant strains as above. The Mo present in the 4.5s protein from the mutants *cnxH* 3 and *niaD* 17 was significantly less than in the wild type enzyme. (Table 3) The 7.8s protein from the *niaD* 8 mutant exhibited near normal amounts of Mo. This particular mutant enzyme lacks not only NADPH-nitrate reductase activity by also reduced benzyl viologen-nitrate reductase

TABLE 3

Molybdenum analysis of NADPH-nitrate reductase and NADPH-cytochrome c reductase from Aspergillus nidulans

Strain	s_{20}, W	Molybdenum nmoles per mg protein	
		Intact* enzyme	H_2O extract of ashed enzyme
bi-1	7.8	5.00	5.32
niaD-8	7.8	4.73	6.03
niaD-17	4.5	0.87	0.91
cnxH-3	4.5	0.09	0.25
cnxE-14	7.8	0.07	0.23

* Purified to a specific activity of 15,000 nmoles nitrite produced or cytochrome c reduced per min. per mg.

activity (4,7). It is the only nitrate inducible, 7.8s NADPH-cytochrome c reductase thus far observed among the six niaD mutants studied. All others produce a constitutive 4.5s NADPH-cytochrome c reductase which is consistantly present in mycelium grown in the absence of nitrate. It is possible that dissociation of this enzyme multimer into the 4.5s and the remaining lighter component(s) by treatment with 8 M urea or phenol:acetic acid:water (1:1:1) will result in the unilateral distribution of Mo with the former. Experiments employing Mo^{99} are in progress in an attempt to answer this question.

Although more enzyme from a greater number of mutants will have to be examined before a definitive conclusion can be set forth, these results provide evidence for the concept that the cnx cofactor mutants are defective in one or more proteins which are essential to the correct biosynthetic acquisition of Mo by the functional native multimer. In the various mutant enzymes examined, the proteins might logically be considered to be the smaller NADPH-cytochrome c reactive protein (4.5s) and the defective, larger 7.8s aggregate.

The latter is consistantly devoid of nitrate reactivity and since it may or may not possess normal levels of Mo, it is not yet appropriate to introduce a model where the metal ion is the basis for subunit assembly. These results affirm the indispensible role of Mo in the nitrate reductase complex.

ACKNOWLEDGMENT

The author thanks Mrs. M. Beaver for superb technical assistance. This work was supported by Public Health Service grant AI 05820 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. Nason, A., A.D. Antoine, P.A. Ketchum, W.A. Frazier III, and D.K. Lee. 1970. Proc. Nat. Acad. Sci., 65:137-144.

2. Ketchum, P.A., H.Y. Cambier, W.A. Frazier III, C.H. Madansky, and A. Nason. 1970. Proc. Nat. Acad. Sci., 66:1016-1023.

3. Downey, R.J. and D.J. Cove. 1971. J. Bact., 106:1047-1049.

4. Downey, R.J. 1972. Microbios. (In press)

5. Wray, J.L. and P. Filner. 1970. Biochem. J., 119:715-725.

6. Cove, D.J. and J.A. Pateman. 1969. Autoregulation of the synthesis of nitrate reductase in Aspergillus nidulans. J. Bacteriol., 97:1374-1378.

7. Arst, Jr., H.N., D.W. MacDonald, and D.J. Cove. 1970. Molec. Gen. Genetics, 108:129-145.

8. Pateman, J.A., D.J. Cove, B.M. Rever, and D.B. Roberts. 1964. Nature, 201: 58-60.

9. Pateman, J.A., B.M. Rever, and D.J. Cove. 1967. Biochem. J., 104:103-111.

10. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. J. Biol. Chem., 193:265-275.

11. Cove, D.J. and B.M. Rever. 1964. Heredity, 19:529.

12. DeLuca, C., M.M. Weber, and N.O. Kaplan. 1956. J. Biol. Chem., 223:559.

13. Huennekens, F.M. and S.P. Felton in S.P. Colowick and N.O. Kaplan (Ed) Methods in enzymology, Vol. III, Academic Press, Inc., New York, 1957, p. 950.

14. Burch, H.B., O.A. Bessey, and O.H. Lowry. 1948. J. Biol. Chem., 175:457-470.

15. Johnson, C.M. and H. Arkley. 1954. Anal. Chem., 26:572-574.

16. Downey, R.J. 1971. J. Bacteriol., 105:759-768.