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Enzymatic and Non-Enzymatic Reduction of Nitrite by Extracts of *Neurospora crassa*

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Two activities causing nitrite disappearance are found in extracts of *Neurospora;* one, inducible by nitrate or nitrite and present only in nitrite-utilizing strains, catalyze the stoichiometric reduction of nitrite to ammonia; the other, present in all strains under all conditions, causes the disappearance of nitrite to something other than ammonia. The latter activity has a molecular weight of about 600 and may contain an oligopeptide, a metal, and an SH group(s). It has no known physiological function.

Most assays of nitrite reductase employ reduced pyridine nucleotides or hydrosulfite plus viologen dyes as electron donors, and the disappearance of nitrite or the oxidation of pyridine nucleotides is measured. Although stoichiometric production of ammonia from the catalytic reduction of nitrite has been shown (4, 7, 9, 11, 13, 18), recovery of less than 85% of nitrite nitrogen in the form of ammonia has been reported (8, 13). In some cases no ammonia was produced (12, 17). A complication arises from the use of reduced pyridine nucleotides as electron donors (13): ammonia is produced nonenzymatically from the degradation of pyridine nucleotides under the alkali microdiffusion conditions employed for the detection of ammonia, and the oxidized form of the pyridine nucleotides is more labile to this type of degradation than the reduced form, though oxidized nicotinamide adenine dinucleotide phosphate may be more resistant to alkali than oxidized nicotinamide adenine dinucleotide (9).

The non-stoichiometric production of ammonia from nitrite reduction catalyzed by crude extracts of wild-type *Neurospora* mycelia induced by nitrite (or nitrate), and the presence of an non-ammonia-producing activity which promotes the disappearance of nitrite, and which accounts for the non-stoichiometry, are examined. The results with non-nitrite-utilizing mutants indicate that the ammonia-producing activity is the physiologically important enzyme.

Neurospora crassa strain 74A was used as the wild-type strain. *Nit-6* is a non-nitrite-utilizing, ammonia-utilizing strain, isolated in our laboratory by treatment of pan-2-B-36A conidia with nitrosoguanidine and subsequently selected by filtration enrichment. Strains *nit-1*, *nit-2*, *nit-3*, *nit-4*, and *nit-5* have been described previously (1, 15). The basic culture medium used was that described by Sorger and Giles (15). Nitrate, nitrite, and ammonia media contain sodium nitrate (2 g/liter), sodium nitrite (0.6 g/liter), and ammonium tartrate (4 g/liter), respectively. *Neurospora* mycelia were cultured without shaking from conidial inocula in ammonia medium at 27 C for 72 h. They were then washed and transferred to designated media for further incubation (8 to 10 h) on a rotary shaker at 27 C. The "induced" mycelial pads were harvested and frozen in liquid nitrogen, before being ground with approximately an equal weight of silica powder and three times the weight of ice-cold 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetate. The slurry was centrifuged at 16,000 $\times$ *g* for 20 min in a refrigerated centrifuge (Sorvall RC-2B). The resulting supernatant was used as the crude extract for enzyme assay.

Nitrite reductase was measured as previously described (3) but using hydrosulfite and methyl viologen instead of hydrosulfite and benzyl viologen as electron donor. At the end of the assay 0.1 ml of 25% barium acetate was added, and the assay mixture was oxidized until colorless by agitation and subsequently centrifuged. Nitrite was measured colorimetrically. Ammonia in the clear supernatant was collected by the Conway microdiffusion method and reacted with Nessler reagent (Paragon, New York), and the resulting color was read at 440 nm in a Beckman DBG spectrophotometer. An assay mixture incubated as the test, but containing no crude extract (or filtrate, or acetone supernatant), or containing no nitrite, was used as a blank in the determination of the nitrite disappearing, or of the ammonia-producing activity,

respectively. Reduced nicotinamide adenine dinucleotide phosphate-nitrate reductase was assayed as described by Subramanian et al. (16). Protein concentration was determined by the Biuret method (5) with bovine serum albumin as standard. One unit of nitrite-disappearing activity, ammonia-producing activity, or nitrate-reductase activity is defined as the disappearance of 1 nmol of nitrite, the appearance of 1 nmol of ammonia, or the appearance of 1 nmol of nitrite per min, respectively.

Crude extracts of wild-type mycelia, which had been exposed to nitrite (or nitrate) for 8 h, catalyzed the non-stoichiometric reduction of nitrite to ammonia. Between 60 and 85% of the nitrite reduced in this way was recovered as ammonia (Table 1). The experiment was repeated more than 10 times, and in each case the result was reproducible in duplicates or triplicates. The lack of stoichiometry was not due to the disappearance of ammonia under the conditions of the assay: when exogenous ammonia was added to the assay mixture at a final concentration of 0.3 mM, 96 to 102% of the added ammonia was recovered at the end of the incubation. Crude extracts of wild-type mycelia exposed for 8 h to ammonia medium or to no-nitrogen medium do not catalyze the reduction of nitrite to ammonia (Table 1). Extracts of *nit-6*, which cannot grow on nitrite or nitrate as sources of nitrogen, but which can assimilate ammonium ions, contain the activity which promotes nitrite disappearance but not the ammonia-producing activity. These results suggest that there may be two activities causing nitrite disappearance; one, present in the wild-type strain and absent in *nit-6*, which is inducible by nitrate and by nitrite, and which catalyze the production of ammonia from nitrite (ammonia-producing activity [AP]); and the other which is present in all extracts, is not inducible, and which converts nitrite to something other than ammonia (nitrite disappearance activity [ND]).

TABLE 1. *Nitrite-reducing and ammonia-producing activities in extracts of wild-type and nit-6 mycelia exposed to different nitrogen sources*[a]

Strains	Nitrogen source in induction medium	Sp act[b]	
		Nitrite reduction	Ammonia production
Wild type	NO_2^-	14.1	8.8
	NO_3^-	24.8	19.3
	NH_4^+	10.9	<0.5
	−N	15.6	0
nit-6A	NO_3^-	11.2	0
	NH_4^+	7.2	0
	−N	12.3	0

[a] Wild-type and *nit-6A* mycelia, pregrown in ammonia media for 72 h, were exposed to different nitrogen sources for 8 h (induction). The mycelia were then harvested, and the extracts were assayed for nitrite-reducing and ammonia-producing activities. The assay time was 10 min.

[b] Units per milligram of protein.

A low-molecular-weight ND was isolated from crude extracts of wild-type mycelia induced on nitrate for 8 h by: (i) ultrafiltration through a Collodion bag (Glaxo, Toronto, Ontario); (ii) precipitation with 50% acetone, the resulting supernatant being lyopholized overnight and resuspended in ice-cold distilled water. The effect of the two isolation procedures is shown in Tables 2 and 3. Both the filtrate from the ultrafiltration and the supernatant from the acetone precipitation contained ND but no AP. The recovery of the ND activity by the two methods was 80% and 70%, respectively.

The difference between nitrite disappearance (30.3 U) and ammonia production (18.0 U), catalyzed by crude extracts of wild-type mycelia which had been exposed to nitrate for 8 h, was apparently due to the activity of the filterable ND (11.5 U) (Table 2). A similar difference, shown in Table 3, was seemingly due to the non-acetone-precipitable ND activity. A crude extract, which was concentrated by ultrafiltration, catalyzed the reduction of nitrite stoichiometrically to ammonia, but the recovery of this activity was only 50% (Table 2). The recovery of

TABLE 2. *Effect of ultrafiltration on the nitrite-reducing and ammonia-producing activities present in crude extracts of wild-type mycelia exposed to nitrate for 8 h*[a]

Preparation	Activity (U/0.4 ml)	
	Nitrite reduction	Ammonia production
Crude extract	29.4	Not done
	30.3	18.0
Crude extract concentrated twofold by ultrafiltration	15.5	n.d.
	17.5	18.8
Filtrate[b]	10.8	n.d.
	11.5	0

[a] Crude extract (10 ml) was filtered through a collodion ultrafiltration membrane. The nitrite-reducing and ammonia-producing activities of 0.4 ml of the untreated extract, of the extract concentrated to 5 ml in the collodion bag, and of the filtrate were assayed.

[b] The activity of the filtrate has been multiplied by 1.25 to account for the 20% loss of filterable activity (see text).

AP in the acetone precipitate was poor, and this activity did not catalyze the stoichiometric production of ammonia from nitrite, possibly because of partial inactivation due to the acetone treatment. The acetone precipitate did not contain active filterable ND activity (Table 3).

TABLE 3. *Filterability of the nitrite-reducing and ammonia-producing activities residing in the supernatant and in the precipitate of fully induced wild-type extract which has been treated with acetone*[a]

Fraction	Activity (U/0.4 ml)		
	Unfiltered		Filtrate nitrite reduction
	Nitrite reduction	Ammonia production	
Crude extract	34.5	16.4	Not done
Precipitate (twofold concn)	18.0	8.6	0 (0)
Supernatant	13.2	<0.5	10.6 (80)[b]

[a] Crude extract (10 ml) was precipitated with an equal volume of acetone at −20 C. The supernatant and the precipitate were freeze dried and subsequently resuspended in 10 and 5 ml of ice-cold distilled water, respectively. Aliquots of the resuspended preparations were passed through a collodion ultrafiltration membrane. Nitrite-reducing and ammonia-producing activities were then measured. Numbers in parentheses represent the percentage of recovery of unfiltered activity.

[b] The activity shown has not been corrected.

The molecular weight of the ND in the supernatant of acetone-treated extracts is estimated to be approximately 600 because of its behavior in a column of Sephadex G-25 (Fig. 1). The activity causing ND was not precipitable by 10% trichloroacetic acid or by 95% ethanol; it was not extractable into nonaqueous solvents such as chloroform or petroleum ether. Pepsin and protease form VIII (Sigma), but not trypsin or protease form VI (Sigma), caused a 50% reduction of the ND after 3 h of incubation at 24 C. The protease-dependent loss of this activity did not seem to be due to nonspecific binding of the ND to proteins, since incubation of the acetone supernatant with bovine serum albumin resulted in no loss of activity. A sulfhydryl group(s) seems to be present in ND. When it was preincubated with iodoacetate in the presence of 10^{-2} M hydrosulfite, there was a 30% loss of activity at a concentration of 10^{-5} M, and a 70% loss of activity at a concentration of 10^{-2} M. *p*-Chloromercurobenzoate at a concentration of 2×10^{-5} M caused a 60% loss of activity under the above conditions. No inactivation was observed when the preincubation took place in the absence of hydrosulfite. A metal seems to be involved in ND. Cyanide, phenanthroline, and bipyridyl, all at 10^{-3} M, had a negligible effect on ND during 2-h prein-

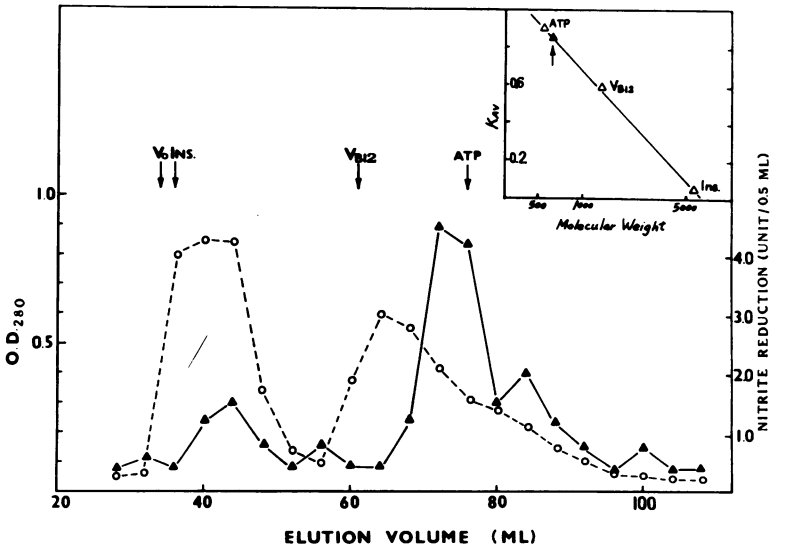

FIG. 1. *Elution profile of the ND activity during filtration through Sephadex G-25. Two milliliters of the supernatant of a crude extract of wild-type mycelia induced on nitrate medium treated with 50% acetone was put onto a column (2.4 by 21 cm) containing 81 ml of Sephadex gel. The eluant was collected in fractions of 4 ml and subsequently assayed for ND activity. A 2-ml mixture containing 1 mg of vitamin B12 (V_{B12}), 2 mg of insulin (Ins.), and 10 mg of adenosine 5'-triphosphate (ATP) was used to standardize the column. The void volume was 34 ml. The inset shows the relationship between molecular weight and Kav value of the markers, and the estimated size of the ND activity. Symbols: ▲, ND activity; O, optical density at 280 nm.*

cubation. In the presence of 10^{-2} M hydrosulfite and an otherwise identical preincubation, cyanide and phenanthroline caused complete inactivation, and bipyridyl caused 60% inactivation.

The filterable ND differs from AP in that it does not catalyze the production of ammonia from the reduction of nitrite. The AP is found in extracts of wild-type, *nit-1*, and *nit-3* mycelia, which are strains that produce activities which cross-react immunologically with nitrate reductase only when these are exposed to nitrite (or nirate) for 8 h (Table 1). The ND is found in extracts of mycelia of all strains of *Neurospora*, including those which do not produce protein which cross-reacts immunologically with nitrate reductase, exposed to any of the nitrogen sources listed in Table 1. Only wild-type, *nit-1*, and *nit-3* strains can utilize nitrite; thus it would seem that ND cannot support growth on nitrite. Extracts of *nit-6*, a strain which can utilize ammonia but not nitrate or nitrite as sole nitrogen sources, has normal nitrate reductase but no AP, suggesting that the latter is required for growth on nitrite or nitrate.

The relatively high specific activity of "nitrite reductase" observed in extracts of mycelia incubated for 17 h with no nitrogen source, compared to that in extracts of mycelia exposed to a medium containing Casamino Acids, which was observed by Cook and Sorger (4), was most probably due to the low protein content of the extracts due to nitrogen starvation of the mycelia (3, 6).

A nitrite-reducing, non-ammonia-producing activity has been reported in extracts of plants (14, 17). The responsible agent was thought to be ascorbic acid. In the present study, the results suggest that the ND activity may be a small polypeptide (molecular weight of approximately 600) containing a sulfhydryl group(s) and a metal(s). The metal may be iron, because we have found that 7.5 μmol of hydrosulfite can reduce 1.5 μmol of nitrite in 20 min in the presence of 0.4 μmol of ferric chloride. In some ways the properties of this nitrite-reducing activity fit the description of siderochromes, which are hexapeptides believed to be responsible for iron transport in *Neurospora* and other microorganisms (10, 19). None of the known siderochromes, however, contain sulfhydryl groups (10).

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