METALLO-ENZYMES IN THE REDUCTION OF NITRITE TO AMMONIA IN *NEUROSPORA*

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It is well established that nitrate reductase, which catalyses the reduction of nitrate to nitrite in some bacteria, fungi and green plants is a molybdo-flavoprotein[1-7]. Although the subsequent reduction pathway from nitrite to ammonia is less clearly defined, the characterisation of nitrite and hydroxylamine reductases in micro-organisms and higher plants[8,9,10] suggests that these compounds are likely to be intermediates in the aliphatic sequence. The recent identification of hyponitrite reductase in *Neurospora*[11] supports the view that the immediate reduction product of nitrite is hyponitrite which is then reduced to hydroxylamine. Nitrite and hydroxylamine reductases are known to be metallo-flavoproteins but hitherto the metals in the enzymes have not been identified. In this paper it is shown that both Cu and Fe are essential for nitrite and hyponitrite reductases and that hydroxylamine reductase is Mn-dependent.

METHODS

Neurospora crassa Wild type 5297a (macroconidial) was grown in a modified Fries culture solution with sodium nitrate as the sole N source. After 4 days growth in the dark at 30°C the felts collected in a Büchner funnel were well washed in glass-distilled water and were frozen at −17°C for 12 h. The felts were then ground in a mortar with four times their weight of 0.1 *M* phosphate buffer (pH 7.5) and then in a Ten Broeck glass macerater at 0°C. The homogenate was centrifuged at 3000 *g* for 15 min at 4°C and the supernatant solution used as the enzyme source. Protein was determined in the extracts by Folin's method. Individual deficiencies of Fe, Cu, Mn, Mo and Zn were produced in *Neurospora* by methods described previously[12].

RESULTS

The results for nitrite, hyponitrite and hydroxylamine reductases determined in the Conway units are given in Table I.

All reagents used were either boiled to eliminate the last traces of ammonia or dispensed in ammonia-free water. Sodium hyponitrite and hydroxylamine hydrochloride were freshly prepared each day. The preparation and determination of sodium hyponitrite has been given elsewhere[11]. The results in Table I show that ammonia is produced enzymically from $NaNO_2$, $Na_2N_2O_2$ or NH_2OH, in the presence of DPNH. The hydroxylamine reductase is particularly active.

The data in Table II indicate that the enzymes are flavin-dependent, since boiled pig heart, FMN, or FAD, stimulated the three enzymes. Their inhibition by atabrine was completely reversed by FAD.

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TABLE I

NITRITE, HYPONITRITE AND HYDROXYLAMINE REDUCTASES

Reductase enzymes	Experiment	mμmoles NH_3 produced in 20 min ml enzyme			Specific activity of enzyme mμmoles NH_3 20 min mg protein
		(1) Complete system	(2) Omit DPNH	(3) Omit enzyme	
Nitrite	1	313	43	0	39
	2	506	69	0	52
	3	504	67	0	57
Hyponitrite	1	187	25	0	23
	2	316	43	0	32
	3	252	34	0	28
Hydroxylamine	1	1378	190	0*	174
	2	2030	280	0	208
	3	1963	270	0	208

Complete system: Outer cell of Conway unit; 0.15 ml 0.2 *M* pyrophosphate buffer (pH 7.5); 0.05 ml boiled pig heart; 0.2 ml enzyme (8 mg protein/ml); 0.1 ml 10^{-2} *M* DPNH and 0.1 ml $4 \cdot 10^{-3}$ *M* $Na_2N_2O_2$ or 0.2 ml $2 \cdot 10^{-3}$ *M* $NaNO_2$ or 0.2 ml $2 \cdot 10^{-1}$ *M* $NH_2OH \cdot HCl$.

Inner cell: 1.5 ml 0.01 *N* HCl.

Incubated for 20 min at laboratory temperature, then 1 ml of saturated K_2CO_3 added to the outer cell and incubated for a further 1 ½ h. 0.4 ml aliquots taken from inner cell for NH_3 determination by the phenol-hypochlorite method.

* When K_2CO_3 was added to hydroxylamine hydrochloride, ammonia was produced non-enzymically. This correction is applied to the data for hydroxylamine reductase in columns (1) and (2).

TABLE II

FLAVIN REQUIREMENTS

Reductase enzymes	(1) Nil	(2) Boiled pig heart	(3) FAD	(4) FMN
Nitrite	16	35	40	35
Hyponitrite	8	16	16	16
Hydroxylamine	57	140	131	140

Enzyme assays as in Table I: (1) omit boiled pig heart; (2) 0.05 ml of boiled pig heart; (3) as (1) plus 0.1 ml 10^{-4} *M* FAD; (4) as (1) plus 0.1 ml 10^{-4} *M* FMN.

The effects of inhibitors on the enzymes are recorded in Table III.

TABLE III

EFFECTS OF GENERAL INHIBITORS

% inhibition of enzyme activity

Reductase enzymes	KCN	2,2'-Diquinolyl	Hydrazine sulphate	"Tetrabase"	1,4-Naphthaquinone	2-Heptyl-4-hydroxyquinoline N-oxide	2,4-Dinitrophenol	p-Chloromercuribenzoate
Nitrite	50	75	100	56	80	100	50	40
Hyponitrite	67	35	78	40	80	100	75	82
Hydroxylamine	32	63	75	55	15	9	0	61

Before assay, 0.2 ml enzymes were incubated for 10 min at room temperature with 0.2 ml of one of the following solutions; 10^{-1} *M* KCN; 10^{-4} *M* 2,2'-diquinolyl; 10^{-1} *M* hydrazine sulphate; 10^{-4} *M* tetramethyldiaminodiphenylmethane ("tetrabase"); 10^{-4} *M* 1,4-naphthaquinone; 10^{-3} *M* 2-heptyl-4-hydroxyquinoline N-oxide in 0.01 *N* NaOH; $4 \cdot 10^{-3}$ *M* 2,4-dinitrophenol; and 10^{-4} *M* *p*-chloromercuribenzoate. Enzymes assayed as in Table I.

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Metal inhibitors reduced the activity of all three enzymes. The depressing effect of naphthaquinone and 2-heptyl-4-hydroxyquinoline N-oxide on the nitrite and hyponitrite reductases suggests the participation of a haem type compound, possibly cytochrome *b*, in the two enzymes. Urethane also inhibited these two enzymes. Dinitrophenol (as well as aureomycin and gramicidin, which are not shown in Table III) inhibited the nitrite and hyponitrite reductases so that possibly a phosphorylation occurs at each of these two steps. The uncoupling reagent had no effect on the hydroxylamine reductase, presumably because in this reduction only a comparatively small energy change is involved. The inhibition of the three enzymes by *p*-chloromercuribenzoate was reversed by glutathione, thus indicating the presence of –SH groups in the three reductases.

TABLE IV

EFFECTS OF METAL DEFICIENCIES

Specific activities of enzymes and growth data as % of normal felts. Enzyme assays as in Table I.

Reductase enzymes	*Omit Fe*	*Omit Cu*	*Omit Mn*	*Omit Mo*	*Omit Zn*
Nitrite	22	36	53	100	68
Hyponitrite	51	53	60	100	100
Hydroxylamine	100	100	57	100	95
Weight of felts	32	43	41	40	59

In considering the effects of metal deficiencies on the three enzymes presented in Table IV, it is clear that any metal deficiency affecting the hydroxylamine or hyponitrite systems would also inhibit nitrite reductase, provided the reductive pathway involved these intermediates. Thus a Mn deficiency which reduced hydroxylamine reductase, also depressed nitrite and hyponitrite reductases, presumably because they are on the same pathway. Deficiencies of either Cu or Fe markedly depressed both nitrite and hyponitrite reductases but hydroxylamine reductase was unchanged. The effects of Cu and Fe deficiencies were also confirmed when nitrite reductase was determined by measuring the disappearance of nitrite[13] instead of ammonia production. The Fe deficiency effect is in agreement with the inhibition of nitrite and hypo-

TABLE V

AN INHIBITOR OF NO_2 REDUCTASE IN Zn-DEFICIENT FELTS

Enzyme units as % of those in the normal felts
(Mean of 4 independent experiments)

(1)	*(2)*	*(3)*	*(4)*	*(5)*	*(6)*
Normal	*omit-Zn*	*(1) & (2)*	*(1) + boiled (2)*	*(2) + boiled (1)*	*(1) + boiled and dialysed (2)*
100	74	82	74	70	100

Enzyme assays as in Table I. In (3) 0.1 ml of the extract from normal felts was mixed with an equal volume of the extract from Zn-deficient felts. In (4) 0.2 ml of (1) was mixed with 0.2 ml of boiled extract from Zn-deficient felts. In (5), 0.2 ml of (2) was mixed with 0.2 ml of boiled extract from normal felts. In (6) as for (4) but the boiled extract from the Zn-deficient felts was dialysed against $10^{-3}M$ pyrophosphate buffer (pH 8) for 12 h before mixing it with an equal volume of the extract from normal felts.

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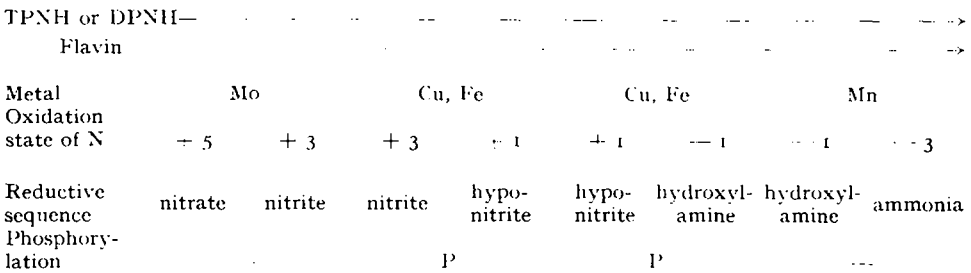
nitrite reductases by the haem inhibitors. It is likely that nitrite and hyponitrite reductases are separate enzymes because they respond differently to a deficiency of Zn, only nitrite reductase being depressed. The reduction product of nitrite reductase, which inhibits the enzyme, is likely to be hyponitrite because the latter markedly reduced the enzyme activity whereas hydroxylamine did not. Hydroxylamine, which was identified as the product of hyponitrite reductase, accumulated in extracts of Mn-deficient felts.

The results in Table V show that there is a dialysable inhibitor of nitrite reductase in Zn-deficient felts.

The aliphatic reduction sequence in *Neurospora* may now be summarised as follows:



Investigations on the role of the metals in these enzymes are now in progress.

SUMMARY

Nitrite, hyponitrite and hydroxylamine reductases in *Neurospora* are shown to be metallo-flavoprotein enzymes; the first two require Fe and Cu and the last one is Mn-dependent, based on metal deficiency and inhibitor studies. Uncoupling reagents depressed nitrite and hyponitrite reductases but were without effect on the hydroxylamine reductase. The aliphatic sequence for the reduction of nitrate is probably nitrate → nitrite → hyponitrite → hydroxylamine → ammonia.

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