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EFFECTS OF A NITRATE REDUCTASE INACTIVATING ENZYME AND NAD(P)H ON THE NITRATE REDUCTASE FROM HIGHER PLANTS AND *NEUROSPORA*

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Summary

Evidence is presented which suggests that the NAD(P)H-cytochrome *c* reductase component of nitrate reductase is the main site of action of the inactivating enzyme. When tested on the nitrate reductase (NADH) from the maize root and scutella, the NADH-cytochrome *c* reductase was inactivated at a greater rate than was the $FADH_2$-nitrate reductase component. With the *Neurospora* nitrate reductase (NADPH) only the NADPH-cytochrome *c* reductase was inactivated. *p*-Chloromercuribenzoate at 50 μM, which gave almost complete inhibition of the NADH-cytochrome *c* reductase fraction of the maize nitrate reductase, had no marked effect on the action of the inactivating enzyme.

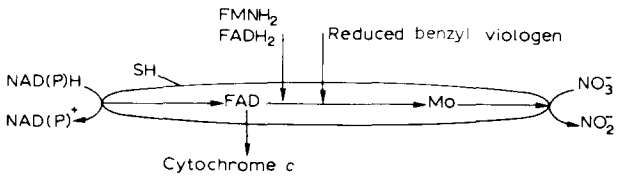
A reversible inactivation of the maize nitrate reductase has been shown to occur during incubation with NAD(P)H. In contrast to the action of the inactivating enzyme, it is the $FADH_2$-nitrate reductase alone which is inactivated. No inactivation of the *Neurospora* nitrate reductase was produced by NAD(P)H alone and also in the presence of FAD. The lack of effect of the inactivating enzyme and NAD(P)H on the $FADH_2$-nitrate reductase of *Neurospora* suggests some difference in its structure or conformation from that of the maize enzyme.

A low level of cyanide (0.4 μM) markedly enhanced the action of NAD(P)H on the maize enzyme. Cyanide at a higher level (6 μM) did give inactivation of the *Neurospora* nitrate reductase in the presence of NADPH and FAD. The maize nitrate reductase, when partially inactivated by NADH and cyanide, was not altered as a substrate for the inactivating enzyme.

The maize root inactivating enzyme was also shown to inactivate the nitrate reductase (NADH) in the pea leaf. It had no effect on the nitrate reductase from either *Pseudomonas denitrificans* or *Nitrobacter agilis*.

Introduction

There is evidence in both fungi [1,2] and higher plants [3,4] that nitrate reductase is an enzyme complex which can be represented as follows:



The NAD(P)H-dependent reduction of nitrate represents the total reaction of the enzyme complex, while the following partial reactions of it can also be measured: a nitrate-inducible NAD(P)H-dependent cytochrome *c* reduction and reduced flavin- or reduced viologen-dependent reduction of nitrate.

A nitrate reductase inactivating enzyme in the mature root of maize seedlings was shown to specifically inactivate the nitrate reductase (NADH) (EC 1.6.6.1) in the maize root and scutella [5]. Inactivation of nitrate reductase (NADH) and $FMNH_2$-nitrate reductase was demonstrated and preliminary studies also suggested inactivation of the nitrate inducible (NADH)-cytochrome *c* reductase. In contrast, reversible inactivation of nitrate reductase in *Chlorella* [6] due to NAD(P)H results in no effect on the nitrate inducible (NADH)-cytochrome *c* reductase component. The inactivating enzyme has been purified 460-fold and estimated to have a molecular weight of 44 000 [7]. Inhibition by phenylmethylsulphonyl fluoride suggests that it is a protease with a serine active site.

In this paper an investigation on the site of action of the inactivating enzyme on the nitrate reductase complex is described. Inactivation of maize root, maize scutella and pea leaf nitrate reductase by NAD(P)H is reported and a comparison made with the action of the inactivating enzyme. The effect of the maize root inactivating enzyme on the nitrate reductase (NADPH) in *Neurospora* (EC 1.6.6.3) and on two bacterial nitrate reductases is also described.

Materials and Methods

Plant material, harvest and extraction procedures

Seeds of *Zea mays* L. (Hybrid Variety DSC1) were supplied by the Dekalb Shand Seed Co., Tamworth, Australia. After surface sterilization by rinsing briefly in 50% (v/v) ethanol followed by 0.05% (w/v) $HgCl_2$ and sterile water, they were grown at 25°C on 1% (w/v) agar containing 5 mM KNO_3, 0.1-strength Hoagland solution and an additional supplement of molybdenum (0.02 μg/ml). The harvest and extraction procedure was as described previously [5].

Preparation and assay of nitrate reductase and the nitrate reductase inactivating enzyme from maize seedlings

Nitrate reductase was isolated from the root tip (0—2 cm of the primary root) and scutellum of 3-day maize seedlings by precipitation from crude ex-

tracts with $(NH_4)_2SO_4$ (40% satn) [7]. This fraction was dissolved in 25 mM phosphate buffer (pH 7.0) containing 1 mM cysteine except when NADH-cytochrome *c* reductase was to be estimated. Assays previously reported for nitrate reductase (NADH) [8], $FAD(H)_2$-nitrate reductase [4] and NADH-cytochrome *c* reductase [4] were employed. For nitrate reductase (NADH), phosphate buffer (pH 7.5) (30 μmol) was used. A similar activity of the plant nitrate reductase was obtained with either $FMN(H)_2$ or $FAD(H)_2$. In the reduced benzylviologen-nitrate reductase assay the flavin was replaced by benzylviologen.

A partially purified sample of nitrate reductase inactivating enzyme (CM32 fraction) was prepared from the mature root of 4-day maize seedlings [7].

Neurospora nitrate reductase

Neurospora crassa (wild type 5297A) was grown on a rotary shaker at 30°C with the medium used by Nicholas and Nason [9]. It was extracted as for the maize root tip but 50% satn $(NH_4)_2SO_4$ was required to give maximum precipitation of nitrate reductase. Nitrate reductase (NADPH), $FAD(H)_2$-nitrate reductase and reduced benzylviologen-nitrate reductase were assayed as described by Garrett and Nason [10]. The assay of NADPH-cytochrome *c* reductase was based on that of Kinsky and McElroy [11] but cyanide was not required.

Pea leaf nitrate reductase

Seedlings of the field pea (*Pisum arvense* L.) were grown on vermiculite watered with a solution containing 5 mM KNO_3, 0.1-strength Hoagland solution and 0.02 μg Mo/ml. The extraction and assay procedure was as for the maize root except no cysteine was employed.

Bacterial nitrate reductase

The nitrate reductase from *Pseudomonas denitrificans* was isolated, partially purified and assayed as described by Radcliffe and Nicholas [12]. Investigations on the nitrate reductase (NADH) of *Nitrobacter agilis* [13] were undertaken on a 44% $(NH_4)_2SO_4$ supernatant fraction supplied by Dr J. Herrera of this Department.

Results

Comparison of the effect of the inactivating enzyme and NADH on maize nitrate reductase

Incubation of the nitrate reductase (NADH) from maize scutella with the inactivating enzyme resulted in loss of activity of the nitrate reductase (NADH), $FAD(H)_2$-nitrate reductase and reduced benzylviologen-nitrate reductase reactions (Table I). It appeared to have a greater effect on the total reaction of the complex nitrate reductase (NADH) than on the partial reactions of it. A low level of inactivation of NADH-cytochrome *c* reductase was apparent. In this case, total NADH-cytochrome *c* reductase was measured and it will be shown later that only a small fraction of this is the nitrate inducible NADH-

TABLE I

EFFECT OF THE INACTIVATING ENZYME AND NADH ON NITRATE REDUCTASE (NADH) AND NADH-CYTOCHROME *c* REDUCTASE

The scutella enzyme sample used contained 0.62 mg protein and the initial enzyme activities were (a) (nmol NO_2^- produced/h):nitrate reductase (NADH), 165; $FAD(H)_2$-nitrate reductase, 79; reduced benzylviologen-nitrate reductase, 53; and (b) (nmol cytochrome *c* reduced/min):NADH-cytochrome *c* reductase, 438. All samples were incubated for 2 h at 25° C.

Treatment	Nitrate reductase (NADH)	$FAD(H)_2$-nitrate reductase	Benzylviologen-nitrate reductase	NADH-cytochrome *c* reductase
None	100	100	100	100
Inactivating enzyme, 10 μg	8	66	59	—
Inactivating enzyme, 20 μg	2	48	22	86
Inactivating enzyme, 40 μg	0	26	0	76
NADH (1 mM)	0	0	0	100

cytochrome *c* reductase. Incubation of the scutella nitrate reductase with NADH caused complete loss of the $FAD(H)_2$-nitrate reductase, reduced benzylviologen-nitrate reductase and hence nitrate reductase (NADH) activities (Table I) but had no effect on NADH-cytochrome *c* reductase activity as reported previously for *Chlorella* [6].

The inactivating enzyme was shown (Fig. 1) to give a more rapid inactivation of nitrate reductase (NADH) than $FAD(H)_2$-nitrate reductase. Following complete loss of nitrate reductase (NADH) activity at 2 h, the $FAD(H)_2$-nitrate reductase reaction was inactivated at an accelerated rate. It is also shown in Fig. 1, as has been reported for other sources of nitrate reductase [3,4], that the nitrate reductase (NADH) reaction is more labile at 25° than $FAD(H)_2$-nitrate reductase. Both were rapidly inactivated at the same rate by NADH

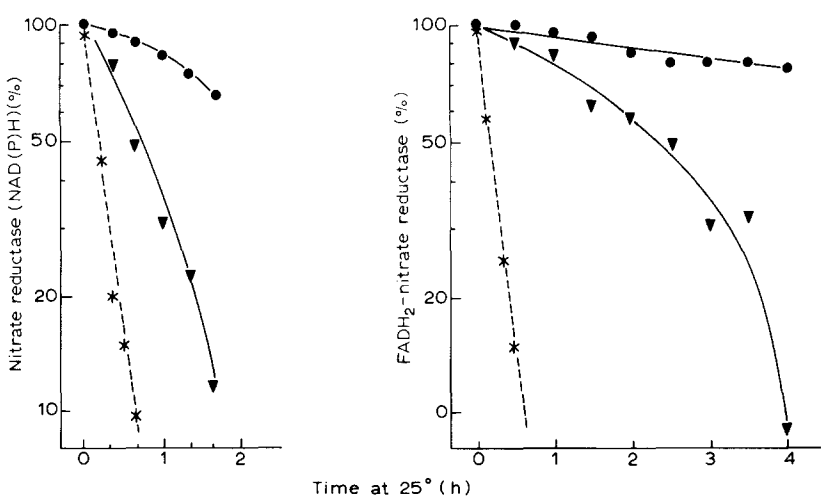


Fig. 1. Inactivation of nitrate reductase (NADH) and $FADH_2$-nitrate reductase by the inactivating enzyme and NADH. A scutella nitrate reductase sample (0.62 mg protein) was incubated at 25°C either alone (●) or with inactivating enzyme (10 μg protein, ▼) or NADH (60 μM, ★). Initial enzyme activities were as in Table I.

TABLE II

FERRICYANIDE REVERSAL OF NITRATE REDUCTASE INACTIVATION

A scutella enzyme sample was used as in Table I but the incubation with ferricyanide and all assays of nitrate reductase were conducted in 90 mM phosphate buffer (pH 7.5).

Treatment	Nitrate reductase (NADH) (nmol NO_2^- produced/h)	
	1 h at 25°C	1 h at 25°C plus 10 min at 25°C with 0.3 mM $K_3Fe(CN)_6$
None	121	101
NADH 0.06 mM	33	87
Inactivating enzyme, 10 μg protein	30	18

(Fig. 1). The response of reduced benzylviologen-nitrate reductase to NADH and the inactivating enzyme was the same as that described for $FAD(H)_2$-nitrate reductase. NADPH at the same concentration was equally effective as NADH on the scutella nitrate reductase but the root-tip nitrate reductase, which in contrast to the scutella enzyme cannot utilize NADPH directly, was much less sensitive to NADPH than NADH. It has been shown in *Chlorella* species [6,14] that the nitrate reductase inactivated by NADH can be reactivated almost immediately by ferricyanide. Ferricyanide can also reverse the inactivation of the maize nitrate reductase by NADH (Table II) but has no influence on the action of the inactivating enzyme. Similarly to *Chlorella* [15], low levels of cyanide markedly enhance the inactivation of the maize nitrate reductase by NAD(P)H, e.g. an 11% inactivation due to 11 μM NADH was increased to 48% by 0.4 μM cyanide.

Nitrate reductase, when partially inactivated by NADH or NADH and cyanide, was apparently not altered as a substrate for the inactivating enzyme

TABLE III

INFLUENCE OF TREATMENT OF NITRATE REDUCTASE WITH NADH AND CYANIDE ON THE ACTION OF THE INACTIVATING ENZYME

A scutella nitrate reductase sample treated as shown was incubated for 1 h at 25°C with and without the inactivating enzyme. The ferricyanide treatment was for 5 min just prior to assay of nitrate reductase. The activity of nitrate reductase measured in the absence of inactivating enzyme is referred to as the initial level. One unit of nitrate reductase corresponds to 1 nmol NO_2^- produced/h. The nitrate reductase-free sample was a scutella sample in which the nitrate reductase had been inactivated by incubation at 25°C.

Treatment	Level of nitrate reductase (units)	Nitrate reductase inactivated	
		Units	%
None	102	39	38
NADH (43 μM)	63	24	38
NADH (201 μM)	23	6	26
NADH (201 μM) + $K_3Fe(CN)_6$ (0.3 mM)	64	23	36
NADH (3 μM) + CN^- (0.4 μM)	63	20	32
Dilution with nitrate reductase (1)	60	30	50
free sample (2)	35	19	55

(Table III). In all cases there was approximately the same percentage loss of nitrate reductase due to the inactivating enzyme as with the untreated enzyme. If the inactivation by NADH or NADH and cyanide reduced the level of nitrate reductase available to the inactivating enzyme, a higher percentage inactivation would have been expected, as shown when lower levels of nitrate reductase were tested (Table III). The reversible thiol inhibitor *p*-chloromercuribenzoate was used to test the effect of blocking the essential thiol group(s) of nitrate reductase on the action of the inactivating enzyme. At a level of 50 μM, which gave almost a complete inhibition of nitrate reductase (NADH) but did not inhibit FAD(H)$_2$-nitrate reductase or reduced benzylviologen nitrate reductase, it was shown to slightly enhance the action of the inactivating enzyme on nitrate reductase (NADH) and reduced benzylviologen-nitrate reductase (10% increase in rate).

Partial isolation of the nitrate-inducible cytochrome c reductase and effect of inactivating enzyme on it

In the barley leaf [4] and in *Neurospora* [11] there is a 3—4-fold increase in the level of NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase, respectively, in the presence of nitrate. The maize root tip and scutella have a relatively high level of constitutive NADH-cytochrome *c* reductase and nitrate results in only a small increase in the level of the enzyme (Table IV). Here a 37% increase in the level of total NADH-cytochrome *c* reductase contrasts with a 25-fold increase in the level of nitrate reductase (NADH). Using a special extraction medium (Table IV) to minimize disruption of cellular components and centrifuging the crude cell-free extract at 272 000 × *g* for 1 h resulted in the nitrate reductase (NADH) remaining in the supernatant fraction while 91% of the NADH-cytochrome *c* reductase recovered was found in the pellet. The fraction of NADH-cytochrome *c* reductase found in the supernatant exhibits a 2.5-fold increase in the root sample from nitrate-grown seedlings and can thus be taken to more accurately represent the nitrate inducible NADH-

TABLE IV

SEPARATION OF A NITRATE-INDUCIBLE NADH-CYTOCHROME *c* REDUCTASE BY CENTRIFUGATION

Root-tip samples of 3-day maize seedlings grown in the presence or absence of 5 mM nitrate were used. The extraction medium was 0.05 M HEPES containing 0.4 M sucrose, 0.1% (w/v) bovine serum albumin, 0.5 mM EDTA, 0.1 mM $MgCl_2$ and 5 mM cysteine (pH 7.5). Enzyme and protein levels were estimated on the 40% $(NH_4)_2SO_4$ precipitate of each fraction.

Fraction	Nitrate in medium	Protein (mg/ml original extract)	NADH-cytochrome *c* reductase (nmol cytochrome *c* reduced/min per mg protein)	Nitrate reductase (NADH) (nmol NO_2^- produced/h per mg protein)
10000 × *g* supernatant	–	0.64	254	5
(10 min)	+	0.60	349	127
272000 × *g* supernatant	–	0.22	10	0
(60 min)	+	0.25	25	172
272000 × *g* pellet	–	0.14	462	0
(60 min)	+	0.16	466	0

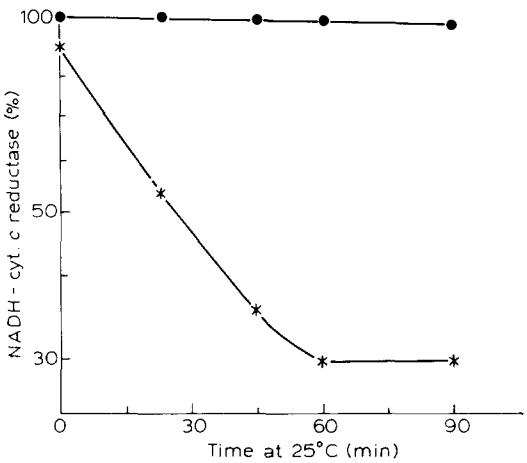


Fig. 2. Influence of the inactivating enzyme on NADH cytochrome *c* reductase from the maize root. The particulate fraction (20 μg protein, •) and soluble fraction (200 μg protein, ⋆) isolated at 272 000 × *g* (Table IV) were tested against 20 μg inactivating enzyme protein.

cytochrome *c* reductase. No increase in activity occurred in the 272 000 × *g* pellet fraction in the presence of nitrate and so this sample represents the constitutive component of NADH-cytochrome *c* reductase.

The effect of the inactivating enzyme on the nitrate-inducible and constitutive NADH-cytochrome *c* reductase fractions of the maize root is shown in Fig. 2. A rapid initial inactivation of the inducible fraction was found but a negligible effect on the constitutive fraction was observed. However, 30% of the soluble fraction was not inactivated, probably due to a non-inducible component also present in the minus-nitrate seedlings. The NADH-cytochrome *c* reductase from minus-nitrate seedlings is not affected by the inactivating enzyme. The rate of inactivation of the nitrate-inducible cytochrome *c* reductase fraction was similar to that shown for the nitrate reductase (NADH) from the scutella (Fig. 1).

Nitrate reductase induced in the presence of tungstate has no nitrate reductase (NADH) activity but an enhanced nitrate inducible NADH-cytochrome *c* reductase activity [4]. When the maize root nitrate reductase complex was modified by a similar treatment with tungstate, it was found that the activity of the inactivating enzyme on the nitrate inducible NADH-cytochrome *c* reductase was not altered.

Action of the inactivating enzyme on Neurospora nitrate reductase

In contrast to the maize seedling enzyme, the nitrate reductase in *Neurospora* is NADPH dependent. A higher level of $(NH_4)_2SO_4$ (50% satn) is required to precipitate it and FAD is a necessary requirement in the assay mixture for the enzyme. The nitrate reductase (NADPH) from *Neurospora* is inactivated by the maize root inactivating enzyme and a higher activity of the latter is observed with *Neurospora* nitrate reductase as substrate than with the root nitrate reductase (Fig. 3). This is probably due to the higher specific activity of the *Neurospora* enzyme and hence lower level of non-nitrate reduc-

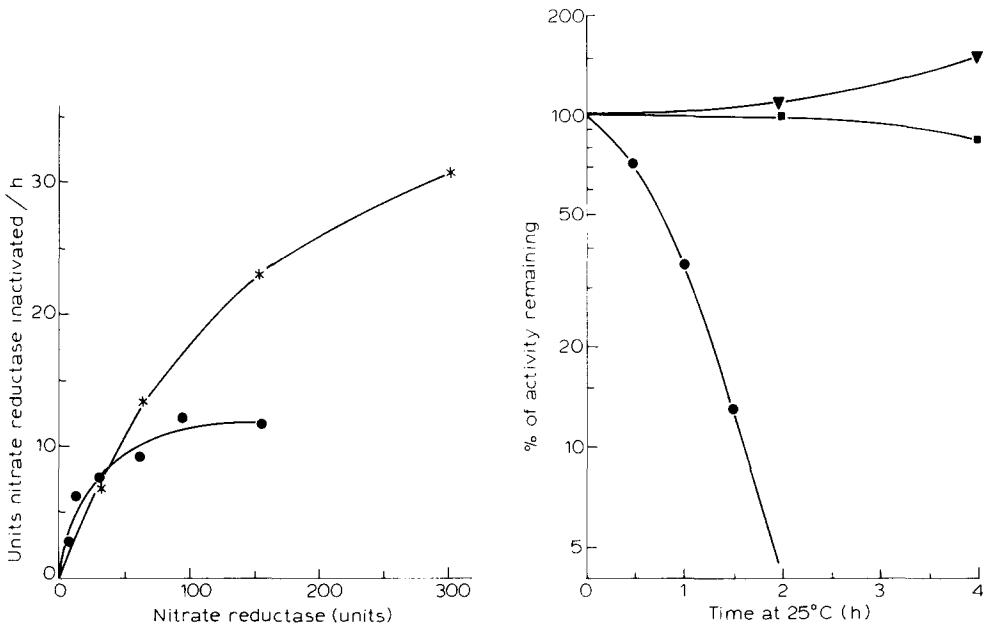


Fig. 3. Inactivation of maize root and *Neurospora* nitrate reductase by the maize root inactivating enzyme. Aliquots of maize root-tip nitrate reductase (NADH) (157 units/mg protein, •) and *N. crassa* nitrate reductase (NADPH) (1224 units/mg protein, ⋆) were incubated with inactivating enzyme (20 μg protein) for 2 h at 25°C.

Fig. 4. Effect of the inactivating enzyme on the *Neurospora* nitrate reductase complex. The fraction of *Neurospora* prepared by 50% satn with $(NH_4)_2SO_4$ (4.02 mg protein) was incubated with maize root inactivating enzyme (0.17 mg protein). The enzyme activities measured and their initial activities were (nmol NO_2^- produced/h per mg protein):nitrate reductase (NADPH), 2716 (•); $FAD(H)_2$-nitrate reductase, 157 (▪) and reduced benzylviologen-nitrate reductase, 865 (▾).

tase protein in the assay of the inactivating enzyme. Protein molecules which inhibit the inactivating enzyme are known to occur in the maize root tip (unpublished). Some evidence was obtained that nitrate reductase from older *Neurospora* cultures was more susceptible to inactivation by the inactivating enzyme.

The *Neurospora* nitrate reductase (50% $(NH_4)_2SO_4$ precipitate) when incubated at 25°C showed some loss of nitrate reductase (NADPH) activity (<20%/h). No change in $FAD(H)_2$-nitrate reductase activity occurred but there was a slight increase in reduced benzylviologen-nitrate reductase. On heating at 49°C for 10 min there was a 5.6-fold increase in reduced benzylviologen-nitrate reductase activity, as reported by Garrett and Nason [10]. Similar treatment of the maize nitrate reductase had no effect on the reduced benzylviologen-nitrate reductase. The effect of the inactivating enzyme on the *Neurospora* nitrate reductase complex is shown in Fig. 4. (Changes in activity described above due to incubation at 25°C have been corrected for.) In the presence of the inactivating enzyme the nitrate reductase (NADPH) is completely inactivated at 2 h, while only a slight loss of $FAD(H)_2$-nitrate reductase has occurred at 4 h, accompanied by a further activation of reduced benzylviologen-nitrate reductase. The reduced benzylviologen-nitrate reductase activated by heating at 49°C

was not influenced by the inactivating enzyme. The nitrate inducible NADPH-cytochrome *c* reductase was activated at the same rate as NADPH-nitrate reductase.

The *Neurospora* nitrate reductase was not inactivated by NADH or NADPH (10—500 μM) tested with and without FAD (16 μM). Cyanide in the presence of NADPH (80 μM) and FAD (16 μM) resulted in an inactivation of the enzyme as reported by Garrett and Greenbaum [16], but the level required (6 μM) was approx. 10-fold that which produced the same inactivation of the maize enzyme in the presence of NADH. No release of cyanide was detected from either the maize scutella or *Neurospora* sample after incubation for 21 h at 30° C. In this study the level of each sample tested was 20-fold that used in the nitrate reductase studies and the procedure used to estimate cyanide [17] was sensitive to about 10 nmol.

Inactivation of pea leaf nitrate reductase

The pea leaf enzyme compared to the other sources of nitrate reductase described above was very labile, especially if cysteine was employed in the extraction or reaction medium. FAD, which had no effect when added to the assay medium, did increase the stability of nitrate reductase (NADH) on incubation at 25° C [18] (Fig. 5). However, even in the presence of FAD, inactivation of nitrate reductase (NADH) was demonstrated with the root inactivating enzyme and NADH ($t_{½}$, 30 min). The $FAD(H)_2$-nitrate reductase and reduced benzylviologen nitrate reductase were also inactivated ($t_{½}$, 120 min).

Influence of the inactivating enzyme and NADH on bacterial nitrate reductase

The following samples of *Ps. denitrificans* were tested: the pellet fraction 144 000 × *g* assayed with NADH or formate and the solubilized pellet fraction

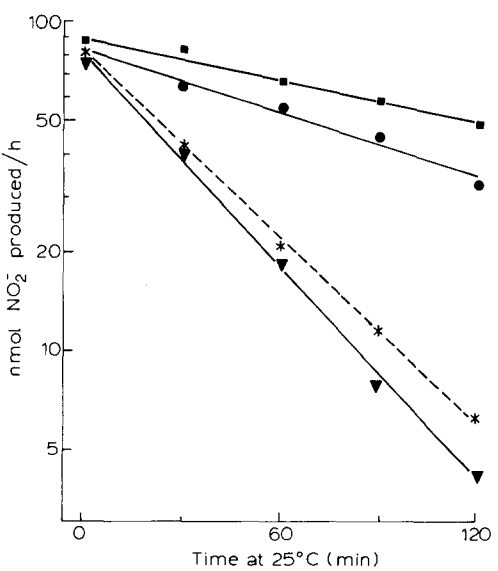


Fig. 5. Effect of FAD, NADH and inactivating enzyme on pea leaf nitrate reductase. A sample of pea leaf nitrate reductase (NADH) (2.30 mg protein) was incubated alone (●), with 0.12 mM FAD (■), with 0.12 mM FAD and inactivating enzyme 76 μg protein (▼) and with 0.12 mM FAD and 0.43 mM NADH (★).

assayed with benzylviologen reduced by *Azotobacter* particles and NADH. Levels of inactivating enzyme and NADH used above had no effect on *Pseudomonas* nitrate reductase. It was also shown that the nitrate reductase (NADH) from *N. agilis* is not influenced by the inactivating enzyme. The *Nitrobacter* reductase, however, is inactivated by NADH [19].

Discussion

The rapid inactivation of the NADH-cytochrome *c* reductase component of the maize nitrate reductase complex suggests that this is the primary site of action of the inactivating enzyme. A slower loss of $FAD(H)_2$-nitrate reductase activity could be due to a conformational change in the enzyme complex following inactivation of the NADH-cytochrome *c* reductase component. When the latter is completely inactivated, loss of $FAD(H)_2$-nitrate reductase activity occurs at an accelerated rate. The inactivating enzyme does not appear to act on the essential thiol group(s) of the NADH-cytochrome *c* reductase component. Cysteine had no influence on its activity and *p*-chloromercuribenzoate had only a slight (stimulatory) influence on the action of the inactivating enzyme. Thus, while both the inactivating enzyme and heat treatment of nitrate reductase result in the preferential loss of activity of the NADH-cytochrome *c* reductase component, a different mechanism of action is suggested for the two treatments based on the protective effect of cysteine against heat inactivation [20].

Induction of nitrate reductase in the presence of tungstate results in an inactive nitrate reductase (NADH) complex with enhanced NADH-cytochrome *c* reductase activity [4]. This treatment did not alter the activity of the inactivating enzyme on the NADH-cytochrome *c* reductase fraction. The inactivation of the nitrate-inducible cytochrome *c* reductase but absence of effect on the constitutive cytochrome *c* reductase emphasizes the specificity of the action of the nitrate reductase inactivating enzyme. It has previously been shown [5] that the inactivating enzyme had no influence on the activity of a range of other enzymes in the maize seedling and on xanthine oxidase, which has a similar flavoprotein structure to nitrate reductase.

The effect of the inactivating enzyme on the nitrate reductase complex is quite distinct from that due to NAD(P)H. In this case there is a rapid reversible inactivation of $FAD(H)_2$-nitrate reductase and hence nitrate reductase (NADH) but no effect on the NADH-cytochrome *c* reductase component. On the basis of such findings, it has been postulated that nitrate reductase in *Chlorella* [6] can exist in an active or inactive form depending on whether the complex is in an oxidized or reduced state. The present work shows that nitrate reductase in the maize root, maize scutella and pea leaf is also inactivated by NAD(P)H. Low levels of cyanide, as reported for the algal cell [15], markedly enhanced this inactivation of the plant enzyme. Partial inactivation of the maize nitrate reductase with NADH or NADH and cyanide did not result in any change of the activity of the inactivating enzyme.

The nitrate reductase in *Neurospora*, although dependent on NADPH, appears to have many similarities to the NADH-specific plant enzyme. The maize root inactivating enzyme caused inactivation of nitrate inducible

NADPH-cytochrome *c* reductase and the NADPH-nitrate reductase reactions of the *Neurospora* enzyme but did not cause a significant inactivation of the $FAD(H)_2$- and reduced benzylviologen-nitrate reductase component. This further suggests that the main site of action of the inactivating enzyme on the nitrate reductase complex is on the NAD(P)H-cytochrome *c* reductase component. The action of the inactivating enzyme on the cytochrome *c* reductase component of the maize and *Neurospora* nitrate reductase but lack of effect on the $FADH_2$-nitrate reductase reaction of the latter confirms the similarity of the cytochrome *c* reductase component in both species but suggests a difference in either the structure or conformation of the $FAD(H)_2$-nitrate reductase component. The *Neurospora* nitrate reductase also differs from the maize enzyme in that NAD(P)H does not cause its inactivation. Since it is considered that the action of the pyridine nucleotides is mediated by low levels of cyanide acting on the reduced form of the enzyme, it could be that the maize sample, in contrast to the *Neurospora* sample, has an endogenous cyanide content. No cyanide release could be detected from either. Alternatively, the inactivation of the maize nitrate reductase (and not the *Neurospora* enzyme) by NAD(P)H may be due to the difference in the structure or conformation of the $FADH_2$-nitrate reductase component of the *Neurospora* enzyme, suggested above by the study on the inactivating enzyme. It is known that the effect of NAD(P)H is on the $FADH_2$-nitrate reductase component of nitrate reductase.

With the pea leaf nitrate reductase it was found that FAD increases the stability of the enzyme during incubation at 25°C. No such effect of FAD was found with the maize root or scutella nitrate reductase and the maize leaf nitrate reductase, even with FAD, was so labile that the inactivating enzyme could not be tested on it. A specific protection by FAD of the nitrate reductase from the spinach leaf has been reported [18]. The action of the inactivating enzyme and NADH on the pea leaf enzyme was not altered by FAD.

The nitrate reductase from *N. agilis* and *Ps. denitrificans* was not influenced by the inativating enzyme from the maize root. This suggests that there is a significant difference between the bacterial nitrate reductase and the enzyme in *Neurospora* and higher plants.

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