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INHIBITION OF THE NITRATE REDUCTASE COMPLEX FROM SPINACH BY OXYLAMINES

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; nitrate reductase complex; oxylamines; inhibition.

Abstract—The nitrate reductase complex from spinach (*Spinacia oleracea*) was found to be inhibited by oxylamine compounds such as aminooxyacetate, hydroxylamine and *O*-methoxylamine. These compounds appear to interact with reduced cytochrome b_{557} during catalysis of the enzyme. However, if the enzyme is maintained in a reduced state by NADH in the absence of nitrate, an additional component involved in $FMNH_2$-nitrate reductase is also affected by them. The binding of the oxylamines with the enzyme is non-covalent in nature as the inhibition can be reversed by treatment with 2-oxoglutarate.

INTRODUCTION

The nitrate reductase complex (NR; EC 1.6.6.1) from higher plants and green algae catalyses the reduction of NO_3^- to NO_2^- using the reductant NADH. The enzyme complex transfers electrons from NADH to NO_3^- via its component sequence FAD, cytochrome b_{557} and molybdenum. *In vitro*, the enzyme shows diaphorase activity which involves the reduction of artificial electron acceptors such as 2,6-dichlorophenol indophenol, ferricyanide [1] and the quinone analogues such as dibromothymoquinone [2]. This partial activity uses NADH as the reductant and involves only the FAD component of the enzyme. The enzyme *in vitro* also reduces NO_3^- to NO_2^- using artificial electron donors such as reduced FMN ($FMNH_2$-NR) and involves only the molybdenum component of the enzyme. The two partial activities, namely the diaphorase and $FMNH_2$-NR, are presumed to be linked by cytochrome b_{557} [3, 4].

Several compounds have been reported to inhibit the two partial activities [1, 5]. Cyanide which inhibits $FMNH_2$–NR reversibly has been assigned physiological significance [1, 6] as the reversibility is dependent on the redox state of the metal component molybdenum. We had previously reported ([7], see also [8]) inhibition of the NR complex by NH_2OH. This inhibition was distinct from other reports in that although NADH-NR was blocked, neither the diaphorase nor the $FMNH_2$-NR function of the enzyme was affected. On this basis we had suggested that NH_2OH may be interfering with the functioning of cytochrome b_{557} essential for linking the two partial activities.

In this paper, we report the inhibition of NADH-NR by aminooxyacetate (AOA), which shares many similarities with the inhibition by NH_2OH. Evidence is presented to demonstrate that the 'oxylamine group' of compounds is responsible for the inhibition observed.

RESULTS

The data presented in Fig. 1 show that in the presence of AOA, NADH–NR was inhibited progressively. Under identical conditions, neither the diaphorase nor the $FMNH_2$-NR activity was inhibited (data not shown). The progressive inhibition suggests that during catalysis the inhibitor binds to the enzyme irreversibly causing increased inhibition. The possibility that AOA may bind to the reduced form of the enzyme is also indicated by the data. This has been confirmed by preincubation experiments and the results are presented in Table 1. Incubation of the enzyme with 1 mM AOA in the presence or absence of NO_3^- alone showed only 5% inhibition. However, the preincubation of the enzyme with AOA in the presence of NADH for 5 min inhibited the enzyme activity by over 90%. Interestingly, the presence of NO_3^- during preincubation along with NADH and AOA relieved the in-

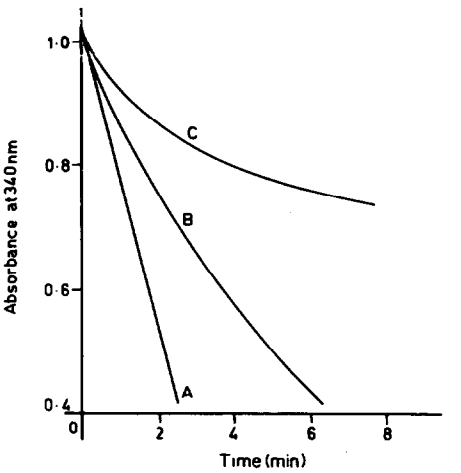

Fig. 1. Effect of aminooxyacetate on NADH-NR. A = 0.00 mM, B = 5 mM and C = 20 mM AOA during the assay. The reaction was followed spectrophotometrically by measuring decrease in *A* at 340 nm.

Table 1. Effect of preincubation of the enzyme with different compounds on the activity of NADH-NR

Preincubation with*	Reaction started by adding†	% Activity
None	NADH, NO_3^-	100
NO_3^-	NADH	100
NO_3^-, NADH	None	100
NADH	NO_3^-	90
NO_3^-, AOA	NADH	95
NADH, AOA	NO_3^-	8
NADH, NO_3^-, AOA	None	71
AOA	NADH, NO_3^-	95

*Nitrate reductase was preincubated for 5 min in a volume of 0.98 ml containing 50 μmol phosphate buffer (pH 7.6) with the following as indicated: NADH, 0.15 μmol; NO_3^-, 10 μmol; AOA, 1 μmol.

†Reaction was started by the addition (20 μl) of the compounds shown.

Nitrate reductase activity was measured spectrophotometrically as given in the Experimental.

Table 2. Effect of aminooxyacetate on nitrate reductase activity on preincubation with NADH

AOA (mM)	% Inhibition NADH-NR	$FMNH_2$-NR
0.01	25	0
0.10	38	6
1.0	92	44
5.0	92	44

Nitrate reductase was preincubated for 5 min with AOA and NADH (0.15 mM) in 50 mM phosphate buffer, pH 7.6. NADH-NR was measured spectrophotometrically after addition of NO_3^- to a final concentration of 10 mM. $FMNH_2$-NR activity was measured for 5 min after appropriate additions of NO_3^-, dithionite and bicarbonate.

Table 3. Effect of hydroxylamine and *O*-methoxylamine on activities of nitrate reductase on preincubation with NADH

$R–ONH_2$ (mM)	% Inhibition NADH-NR	$FMNH_2$-NR
Hydroxylamine		
0.05	58	14
0.10	78	30
0.50	90	50
O-Methoxylamine		
1	20	0
5	27	0
10	44	10
40	85	25

Preincubation and the assay were carried out as given in Table 2.

hibition. The results show that the reduced enzyme was inactivated readily by AOA. The protection provided by NO_3^- can be easily understood if we consider that both NO_3^- and AOA compete for the reduced enzyme. The progressive inhibition observed in Fig. 1 can also be explained on this basis. This behaviour of AOA was identical to NH_2OH and can be interpreted in a manner similar to that which we stated earlier for NH_2OH [7]. We wish to mention that during preincubation of the enzyme with NADH and AOA, NADH was not destroyed but remained unutilized.

Preincubation of AOA with enzyme in the presence of NADH did, to some extent, inhibit $FMNH_2$-NR. As shown in Table 2, at 1 mM AOA, the $FMNH_2$-NR activity was inhibited by 44%, whereas NADH-NR was inhibited by over 90%. Increased concentration of AOA did not result in increased inhibition of $FMNH_2$-NR. The inhibition of $FMNH_2$-NR observed under preincubation conditions was similar to the NH_2OH effect as shown later.

The similarity in the inhibitory effects of NH_2OH and AOA and the presence of 'oxylamine' ($–ONH_2$) in both these compounds suggested the possibility that the group '$–ONH_2$' may be responsible for the inhibition. If this was true, we argued that *O*-methoxylamine ($Me–O–NH_2$) would also inhibit NADH-NR and $FMNH_2$-NR on preincubation of the enzyme in the presence of NADH and *O*-methoxylamine. The data presented in Table 3 show that both NH_2OH and $Me–O–NH_2$ inhibited NADH-NR to a great extent and $FMNH_2$-NR to some extent. Compounds like methylamine, propylamine or benzylamine which contain the $–NH_2$ group but not the $–ONH_2$ group did not inhibit the enzyme even after preincubation with NADH (data not presented). A comparison of the inhibitory effects of NH_2OH, AOA and *O*-methoxylamine shows that NH_2OH was the most potent inhibitor and *O*-methoxylamine was the weakest one. None of these compounds showed more than 50% inhibition of the $FMNH_2$-NR activity.

The enzyme inhibited by NH_2OH and AOA (as also *O*-methoxylamine) could not be fully reactivated by passing through G-25 or treatment with ferricyanide. As presented in Table 4, potassium ferricyanide reactivated the enzyme to *ca* 20% only. Previously, reactivation of NH_2OH inhibited NR by KNO_3 was reported by Solomonson and Vennesland [9]. In their experiments, the NH_2OH concentration used was much lower than the one used here. Reactivation was incomplete. Subsequently, Solomonson [10] attempted reactivation of NH_2OH inhibited enzyme by ferricyanide, but the reversibility observed was incomplete. More recently, Hucklesby *et al.* [8] have reported partial reactivation of the NH_2OH inhibited enzyme by ferricyanide. In their experiments reactivation to the extent of 20% was observed. Our results on partial reactivation are similar to those of Hucklesby *et al.* [8]. Considerable reactivation in our studies was observed by addition of 100 mM 2-oxoglutarate (Table 4). Over 70% of the original activity was restored.

Table 4. Reactivation of the oxylamine inactivated nitrate reductase

	Activity (% of control)		
	No addition	Potassium ferricyanide	2-Oxoglutarate
Hydroxylamine	12	20	72
Aminooxyacetic acid	8	18	74

Nitrate reductase was incubated with NADH (0.15 mM) and oxylamine (1 mM) in phosphate buffer, pH 7.6 (50 mM), for 5 min. It was then chromatographed on a Sephadex G-25 column equilibrated with phosphate buffer, pH 7.6 (50 mM), to remove NADH and oxylamines. The protein fraction with the highest absorbance at 280 nm was used as inactivated nitrate reductase.

Inactivated nitrate reductase was preincubated with either 0.1 μmol ferricyanide or 10 μmol 2-oxoglutarate in a volume of 0.1 ml. At the end of 20 min, the NADH-NR activity was assayed by adding 0.9 ml of assay mixture containing phosphate buffer, pH 7.6 (50 μmol); FAD, 0.02 μmol; NADH, 0.15 μmol; and NO_3^-, 10 μmol. The activity was measured by colorimetric estimation of NO_2^- as given in the Experimental. The activities were converted to nmol of NO_2^- produced per unit A at 280 nm for comparison purposes.

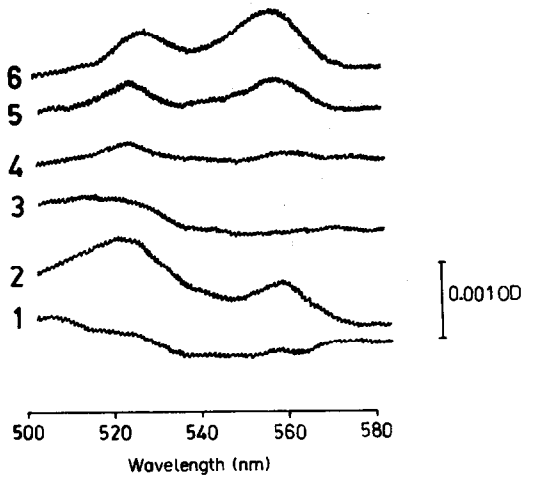

Fig. 2. Difference spectra of the nitrate reductase complex under various conditions. The enzyme protein (5 mg) was taken in 1 ml potassium phosphate buffer which contained 7.5% glycerol (carried over with the enzyme). Where mentioned, compounds in a volume of 5 μl were added to the sample cuvette to give a desired concentration. The difference spectra were recorded using a split beam spectrophotometer. One ml cuvettes were employed. (1) No addition; (2) NADH (0.15 mM) added to the sample cuvette; (3) NO_3^- (10 mM) added to the sample cuvette from (2); (4) NH_2OH (1 mM) added to the sample cuvette from (3); (5) NADH (0.15 mM) added to the sample cuvette from (4); (6) same as in (5) but after 5 min. In all cases, buffer equivalent to the amount of solution added to the sample cuvette was added to the reference cuvette to maintain an equal volume in both cuvettes.

The data presented in this paper on AOA and earlier [7] on NH_2OH suggested that one of the sites on the enzyme with which these inhibitors could be interacting relates to cytochrome b_{557}. In order to check whether the reduction of cytochrome b_{557} is blocked by NH_2OH, room temperature difference spectra of the enzyme were studied under various conditions using a split beam spectrophotometer. The difference spectra presented in Fig. 2 show that addition of NADH to the sample cuvette induced an increase in absorbance at *ca* 525 and 557 nm (cf. curves 1 and 2). This indicates the reduction of cytochrome b_{557} by NADH. Addition of NO_3^- to this sample (containing NADH) decreased the peaks observed at 525 and 557 nm with time indicating the oxidation of cytochrome b_{557} by NO_3^- (cf. curve 3 with 2). Addition of NH_2OH to the sample containing NO_3^- and NADH showed a small increase at 525 and 557 nm (curve 4) which increased further on addition of NADH (cf. curves 5 and 6 with 4). This shows that in the presence of NH_2OH, reduction of cytochrome b_{557} is not blocked, but its oxidation by NO_3^- seems to be inhibited. The peak positions observed by us for reduced cytochrome b_{557} were similar to those reported by Notton *et al.* [11] and Somers *et al.* [12]. Considering the preincubation experiments with NADH, it appears that NH_2OH forms an adduct to reduced Fe (Fe^{2+}) in the haem and that this is not easily reversed by the presence of Mo (IV).

DISCUSSION

The evidence presented in the earlier paper [7] and in this paper shows that NH_2OH or AOA interacts with the nitrate reductase at two places, depending upon the conditions. (A) Interaction with cytochrome b_{557}: In the presence of NADH and NO_3^-, NH_2OH or AOA blocks electron transport only at cytochrome b_{557}. Neither the NADH dehydrogenase nor the $FMNH_2$–NR activity is affected (data not presented). The difference spectra presented in Fig. 2 suggest that the reduction of cytochrome b_{557} is not affected, but that its oxidation is, in the presence of NH_2OH. In an earlier paper [7], we discussed how, during the turnover of the enzyme, NH_2OH can interact with reduced cytochrome b_{557} leading to progressive inhibition.

(B) Interaction with a component of $FMNH_2$-NR: Data presented in Tables 2 and 3 demonstrate that besides cytochrome b_{557}, NH_2OH or AOA must be interacting with a component of $FMHH_2$-NR when the enzyme is preincubated with NADH and the inhibitor. One assumption is made while drawing this conclusion. It is that when $FMNH_2$-NR is assayed, the electrons from $FMNH_2$ to NO_3^- are transferred directly through molybdenum and that the functioning of cytochrome b_{557} is not essential. This assumption is valid because $FMNH_2$-NR is unaffected, although the cytochrome b_{557} function is affected when the enzyme is preincubated with NADH and NH_2OH, or AOA in the presence of NO_3^-. Considering the fact that NH_2OH or AOA must be interacting with a component of $FMNH_2$-NR in the absence of NO_3^- but the presence of NADH, it appears that for such an interaction to occur both cytochrome b_{557} and molybdenum should be in a reduced state. The exact component of $FMNH_2$-NR whose function is blocked by the inhibitor is not known, although it is tempting to invoke reduced molybdenum. However, the inability of ferricyanide to reactivate fully such an inhibited enzyme

(data not presented) suggests that the component involved may be some other than molybdenum.

In our earlier publication, we had reported that NH_2OH did not inhibit $FMNH_2$-NR, even on preincubation of the enzyme from *Amaranthus* with NADH in the absence of NO_3^- [7]. The difference between the *Amaranthus* enzyme and spinach enzyme could be a species difference. Alternatively, the difference could be due to the ratio of $FMNH_2$-NR to NADH-NR in the enzyme preparation. We favour the latter possibility because in a spinach enzyme preparation that had a higher ratio of $FMNH_2$-NR or NADH-NR than was present in the *Amaranthus* enzyme (unpublished data) we did not see inhibition of $FMNH_2$-NR by NH_2OH on preincubation with NADH alone.

The inhibition of NR by NH_2OH, AOA and *O*-methoxylamine (all containing an $-ONH_2$ group) but not by other primary amines (not containing $-ONH_2$) supports the conclusion that the $-ONH_2$ group of these compounds interacts with the reduced cytochrome b_{557}.

The NH_2OH inactivated NADH–NR could be reactivated by 2-oxoglutaric acid (Table 4). It is known that 2-oxoglutaric acid (2-OG) and other keto acids react with NH_2OH and AOA and that they reverse the inhibition caused by these compounds [13]. The activation of the NH_2OH inactivated enzyme by 2-OG shows that NH_2OH binding to the enzyme is not covalent, but a tight binding.

EXPERIMENTAL

Freshly harvested spinach (*Spinacia oleracea*) leaves served as the source of enzyme. The leaves were deribbed and washed with H_2O before use. All isolation procedures were carried out at 4°.

Enzyme purification. NR was extracted in 3 vol./wt ratio of 0.1 M KPi buffer (pH 7.5) with 1 mM EDTA, and partially purified using protamine sulphate, $(NH_4)_2SO_4$ and DEAE-cellulose [2]. The enzyme was stored at −20° in separate small vials. The enzyme was desalted on a Sephadex G-25 column equilibrated with extraction buffer before use. The partially purified enzyme had a sp. act. of 50–70 nkat/mg protein with a ratio of $FMNH_2$-NR to NADH-NR of 1.2.

Protein concn was estimated spectrophotometrically [14].

Enzyme assays. NADH-NR was assayed in a final vol. of 1 ml containing 50 μmol KPi buffer (pH 7.6), 10 μmol NO_3^-, 20 nmol FAD, 150 nmol NADH and enzyme. The activity was estimated either by following the rate of disappearance of NADH spectrophotometrically or by a colorimetric method as in ref. [7]. $FMNH_2$-NR and diaphorase activities were estimated by the method described in ref. [7].

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