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HYDROXYLAMINE INHIBITION OF THE NITRATE REDUCTASE COMPLEX FROM *AMARANTHUS*

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Key Word Index—*Amaranthus viridis*; Amaranthaceae; nitrate reductase complex; hydroxylamine; inhibition kinetics.

Abstract—Nitrate reductase from *Amaranthus viridis* is similar to nitrate reductase from other plant sources. NH_2OH inhibits nitrate reduction from NADH by the nitrate reductase complex, but it does not inhibit either the NADH-dehydrogenase activity or nitrate reduction from reduced flavin mononucleotides. The inhibition observed was non-competitive with nitrate when the enzyme was pre-incubated with NH_2OH and NADH, and competitive with nitrate without pre-incubation. The K_i values for NH_2OH were 5 μM and 30 μM with or without pre-incubation respectively.

INTRODUCTION

The plant nitrate reductase (NR) complex (EC 1.6.6.1.), performs 3 catalytic reactions [1] viz. NADH–nitrate reductase activity (NADH–NR), NADH-dehydrogenase activity and NO_3^- reduction from reduced flavin mononucleotide (FNH_2–NR). Inhibitor studies have shown that NADH-dehydrogenase activity and FNH_2–NR are 2 different functional moieties and NADH–NR includes both the moieties. NADH-dehydrogenase activity is inactivated when heated to 45° in the absence of FAD and by some sulphydryl-reactive inhibitors [1–3], but is not inhibited by cyanide [3]. FNH_2–NR however is inactivated by cyanide and azide [1, 3]. The inhibition by cyanide is enhanced on pre-incubation with NADH [3].

The final product of NO_3^- assimilation, viz. NH_4^+ does not inhibit NR; however, NO_2^- inhibits NR competitively with a K_i higher than the K_m for NO_3^- [4]. NH_2OH has been reported to be a product of *in vitro* NO_2^- reduction in the presence of NH_4Cl but precise experimental details of the work and data have not been provided [5]. NH_2OH inhibits NR from *Chlorella* [4] and *Neurospora crassa* [6]. However, there are no reports on the inhibition of NR by NH_2OH from plant sources. Hence we undertook studies to find out whether NR from plant sources is affected by NH_2OH and if so what the characteristics of inhibition are. The data obtained clearly indicate that NH_2OH is a potent inhibitor of NADH–NR activity, but that it does not affect dehydrogenase or FNH_2–NR activity of the enzyme.

RESULTS

The effect of different concentrations of NH_2OH on the 3 types of activities of the NR complex is shown in Table 1. NADH–NR activity is reduced to almost 50% at 0.1 mM NH_2OH concentration, whereas FNH_2–NR activity was not affected even at 1 mM NH_2OH. At very high concentrations (10 mM) almost 50% reduction in the FNH_2–NR activity was observed. The NADH-dehydrogenase activity was not affected by NH_2OH, even at high concentrations.

Table 1. Effect of NH_2OH on the activities of the NR complex

NH_2OH (mM)	% activity		
	NADH–NR	FNH_2–NR	NADH-dehydrogenase
0	100	100	100
0.01	87	117	100
0.1	52	110	100
1	17	96	100
10	4	56	110

The reaction was started with the addition of enzyme. The assays were conducted as described under Experimental.

The time dependent inhibition of NADH–NR by 0.5 mM NH_2OH is shown in Fig. 1. Activity increased linearly with time up to 30 min in the absence of NH_2OH. However, in the presence of NH_2OH the inhibition increased progressively.

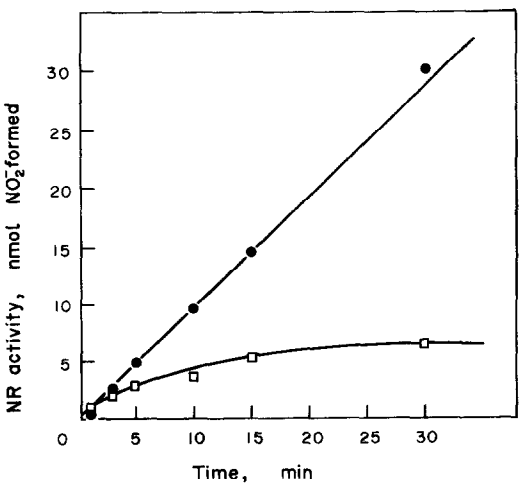

Fig. 1. Effect of NH_2OH on the NR activity with time. (●) No NH_2OH, (□) 0.5 mM NH_2OH.

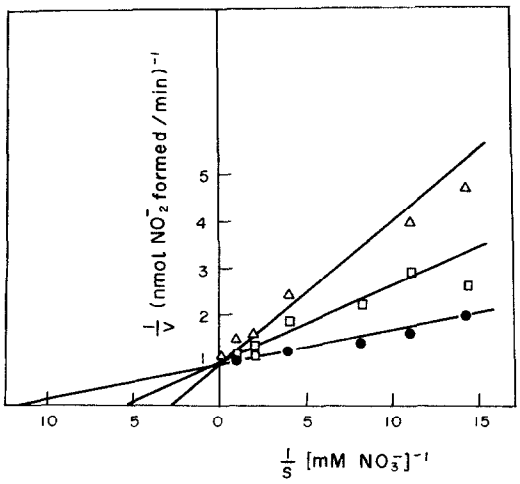

Fig 2. Inhibition of NR by NH_2OH with NO_3^- as variable substrate (Lineweaver–Burk plot). (●) No NH_2OH; (□) 0.05 mM NH_2OH (△) 0.15 mM NH_2OH.

Table 2 Effect on NADH–NR activity of the NR complex on pre-incubation with different compounds

Compounds	Reaction started by adding	% activity
None	NADH + NO_3^-	100
NADH	NO_3^-	90
NO_3^-	NADH	100
NH_2OH	NO_3^- + NADH	52
NO_3^- + NH_2OH	NADH	48
NADH + NH_2OH	NO_3^-	9

Enzyme (2 to 4 mg) was incubated and at the end of 15 min the reaction was started by adding rest of the components of the assay system. The concentrations used in mM: NO_3^-, 2.0: NADH, 0.37: NH_2OH, 0.1.

When the NADH–NR assay was performed by starting the reaction with the addition of NADH, the inhibition by NH_2OH was apparently fully competitive (Fig. 2). This is, however, not a truly competitive type of inhibition as discussed later. The K_i value was 0.03 mM. The Hill plot showed a Hill coefficient slightly less than one, for both NO_3^- and NH_2OH. The K_m for NO_3^- in the absence of NH_2OH was *ca* 0.1 mM which is similar to that reported by several other workers [3, 7].

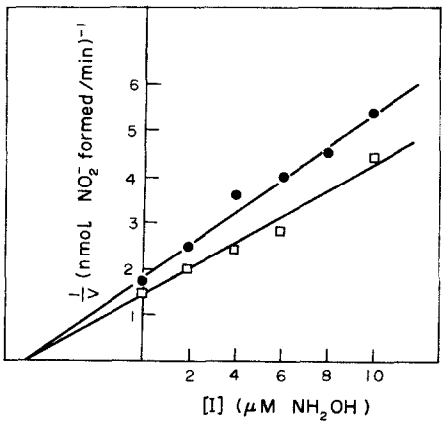

Fig. 3. Inhibition of NR by NH_2OH, on pre-incubation with NADH (0.37 mM) for 15 min, with variable NH_2OH concentration (Dixon plot). (□) 2 mM NO_3^-; (●) 0.5 mM NO_3^-

Table 3. Effect of NH_2OH on the NR complex on pre-incubation with NADH

NH_2OH (mM)	% activity NADH–NR	FNH_2–NR	NADH–dehydrogenase
0	100	100	100
0.01	14	135	100
0.1	9	134	100
1	0	80	100

The reaction was started by adding NO_3^- for NADH–NR activity after pre-incubation of the enzyme protein with NADH and NH_2OH. In FNH_2–NR assay, after pre-incubation of the enzyme protein with NADH and NH_2OH, the reaction was started by adding appropriate amounts of dithionite, bicarbonate, FMN and NO_3^-, as given in Experimental. In this case the interference by NADH on the colour development was overcome by PMS treatment. In the dehydrogenase assay, the reaction was started by adding DCIP after pre-incubation of enzyme with NADH and NH_2OH for 15 min.

From Fig. 1 it was apparent that the inhibition by NH_2OH increased with time. Hence the effect of pre-incubation of the enzyme in presence of NADH or NO_3^- with NH_2OH was studied (see Table 2). The inhibitory effect of NH_2OH is considerably enhanced on pre-incubation of the enzyme with NH_2OH in the presence of NADH. Pre-incubation in the absence of NADH did not show this effect. It therefore appears that the reduced form acquired by the enzyme in the presence of NADH facilitates NH_2OH binding. The type of inhibition effected by NH_2OH, when pre-incubated in the presence of NADH is non-competitive in nature (see Fig. 3). The K_i was *ca* 5 μM. The Hill plots also gave a coefficient less than one in this case.

The effect of pre-incubation of the enzyme with NH_2OH in the presence of NADH was studied (Table 3). NADH–dehydrogenase activity is not inhibited even at the concentrations of NH_2OH where NADH–NR is totally inhibited. It is interesting to note some stimulation at lower concentrations on FNH_2–NR activity.

Cyanide is known to be an inhibitor of both NADH–NR and FNH_2–NR activities. Results given in Table 4 show that both types of activities are almost identically affected by CN^- in the enzyme isolated from *Amaranthus*.

DISCUSSION

The NR from *Amaranthus* is inhibited by NH_2OH, like the NR from *Chlorella* [4] and *Neurospora crassa* [6]. The NR from *Amaranthus* when assayed by adding NADH, NH_2OH and NO_3^- simultaneously seems to be

Table 4. Effect of CN^- on the activities of NR complex

CN^- (mM)	%activity NADH–NR	FNH_2–NR
0	100	100
0.01	85	88
0.05	37	44
0	12	27

competitively inhibited by NH_2OH with a K_i value of 30 μM. However, if the enzyme is pre-incubated with NH_2OH in the presence of NADH the inhibitory effect is considerably enhanced and the K_i under these conditions is reduced to *ca.* 5 μM. The type of inhibition on pre-incubation changes to noncompetitive. In these respects the action of NH_2OH seems to be very similar to that of CN^- [1, 3, 4, 8]. Our studies with CN^- confirm the earlier findings of Relimpio *et al.* [3]. CN^- inhibits FNH_2–NR activity, leaving the dehydrogenase function intact. NH_2OH similarly does not inhibit the dehydrogenase (as assayed by 2,6-dichlorophenol indophenol or cyt reduction) function; however NH_2OH is not inhibitory to the FNH_2–NR function. From this it appears that unlike CN^-, NH_2OH may not be interfering with the molybdenum (Mo) function of NR. If we accept the proposal [9, 10] that in the NR complex, cytochrome (*b*-557) links the NADH–flavin moiety (responsible for dehydrogenase activity) with the Mo moiety (responsible for FNH_2–NR activity), our results can be best interpreted by assuming that NH_2OH interferes with the cytochrome functioning. *Chlorella* NR has been shown to possess a cytochrome which can be reduced by NADH and oxidized by NO_3^- [10]. Spinach NR has also been shown to contain a *b*-type cytochrome [11].

When both NO_3^- and NADH are present, the cyt *b*-557 is mostly in the oxidized state, i.e. Fe^{3+} [9, 11], and therefore the terminal Mo is also oxidised. If we assume that NH_2OH can bind only to the reduced cyt *b*-557 (Fe^{2+}) irreversibly, the competitive and non-competitive kinetics observed could be explained. Thus, if NH_2OH is reacting with the reduced cyt *b*-557 (Fe^{2+}), it can react with the enzyme only during its turnover before Mo can cause re-oxidation of haem. But since the concentration of NO_3^- determines the proportion of oxidized Mo, NH_2OH in effect is competing with NO_3^- indirectly. Therefore, the inhibition by NH_2OH cannot be termed purely competitive but rather as partially competitive. Even the term partially competitive is not strictly correct because NH_2OH does not seem to alter the binding affinity of the enzyme for NO_3^- by binding at a different site as is evident from the fact that NO_3^- reduction from reduced Mo is unaffected by NH_2OH (see Table 3, FNH_2–NR activity). We would, therefore, consider the inhibition by NH_2OH as apparently competitive with respect to NO_3^-. Once NH_2OH binds to the enzyme NO_3^- cannot be reduced at all by NADH because Mo would not be in a reduced state. This is the result of a block at the cytochrome level by NH_2OH. Hence, when the enzyme is pre-incubated with NADH and NH_2OH, a typical non-competitive inhibition is observed. When NH_2OH is present during assay (but without preincubation), NH_2OH binds progressively to the enzyme and inactivates it. This explains the increase in the inhibition of NR with time by NH_2OH (Fig. 1). At higher NO_3^- concentrations the steady state level of the reduced cyt *b*-557 is lower and hence the probability of NH_2OH binding to the enzyme and inactivating it is reduced. This is why at higher NO_3^- concentrations the inhibition by NH_2OH is less and vice versa.

Loussaert and Hageman [5] have recently concluded that NH_2OH is the end product of NO_2^- reduction in the presence of NH_4Cl *in vitro*. In view of this it is tempting to suggest a physiological significance to the inhibition of NR by NH_2OH. This is particularly so because the inhibition is brought about at very low NH_2OH concentrations. However, to suggest a physiological role, further information is needed. Thus, we do not know if NH_2OH under certain conditions released from the enzyme into the medium, can leach out of the chloroplast (since NO_2^- reductase is in the chloroplast) or can be bound to NR which is in the cytoplasm. An indirect regulatory role of NH_2OH in *Chlorella* has been suggested by Solomonson and Spehar [12]. These authors believe that NH_2OH in the presence of glyoxylate through enzymatic reactions can ultimately produce HCN. The HCN has been invoked as the regulator of NR. We wish to emphasize here that since NH_2OH seems to bind to the NR at a place different from either CN^- or any other inhibitor known so far, it may prove useful in obtaining more information regarding the electron transport and functioning of NR complex.

EXPERIMENTAL

Leaves of *Amaranthus viridis* collected from the field served as the source of the enzyme.

Enzyme purification. The enzyme was isolated and purified partially according to ref. [13]. The activities obtained in the Sephadex G-25 fraction ranged between 5 and 10 nmol/min/mg protein and was used in the present studies.

Enzyme assays. NADH–NR was measured in a final vol of 1 ml containing 15 μmol of Pi buffer (pH 7), 2 μmol of NO_3^-, 0.02 μmol of FAD, 0.37 μmol of NADH and enzyme. The reaction was started with the addition of NADH unless otherwise mentioned and after 15 min at 26°, the reaction was terminated with the addition of 0.1 ml of 1 M ZnOAc [13]. After centrifugation for 5 min at 100 *g*, 1 ml aliquot of the supernatant was treated with 5 nmol of phenazinemethosulphate for 20 min in the dark [14]. The NO_2^- was assayed colorimetrically by the procedure of ref. [15].

In case of FNH_2–NR assay, the Py nucleotide was replaced by 0.2 μmol of FMN, together with 0.8 mg $Na_2S_2O_4$ and 0.8 mg $NaHCO_3$ [16]. The reaction was terminated by vigorous shaking in vortex mixer to oxidize FNH_2 and then by the addition of 0.1 ml of 1 M ZnOAc. Before the estimation of NO_2^-, 0.1 ml of HCHO (0.74%) containing 0.1% triton X-100 was added to the supernatant [17].

NADH–dehydrogenase activity was measured by following the reduction of DCIP (0.1 mM) at 600 nm and cyt *c* (1 mg/ml) at 553 nm.

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