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STABILITY OF NITRATE REDUCTASE AND SOURCE OF ITS REDUCTANT IN *SORGHUM* SEEDLINGS

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Key Word Index—*Sorghum bicolor*; Gramineae; nitrate reductase; effect of NADH; stability; source of NADH.

Abstract—Pre-incubation of nitrate reductase from *Sorghum* seedlings with NADH increased enzyme activity by 25%. Ferricyanide had no effect. NADH protected the enzyme from inactivation during storage. Malonate inhibited *in vivo* nitrate reduction in *Sorghum* leaves by 95%. The inhibitory effect of malonate was reversed by fumarate. Sodium fluoride in the presence of phosphate also inhibited *in vivo* nitrate reduction by 60%. It is suggested that NADH generated via the citric acid cycle is utilized for nitrate reduction in *Sorghum* seedlings.

INTRODUCTION

Nitrate reductase (NR) from a variety of sources has been shown to be regulated by pyridine nucleotides [1]. In *Chlorella fusca*, NR can be reversibly converted into an inactive form by reduction with NADH in the presence of ADP [2, 3]. Lorimer *et al.* [4] and Solomonson [5] have shown that inactivation of NR by cyanide is stimulated by NADH and the inactive enzyme is reactivated by oxidation with ferricyanide. On the contrary, our earlier results have shown that NR from rice and wheat leaves is activated by NADH [6, 7]. In addition to this, NADH also prevents the loss of enzyme activity in cell-free extract [7]. Recent studies of Dunn-Coleman and Pateman [8] with cell-free extracts of *Aspergillus nidulans* have shown that *in vitro* loss of NR can be reversed by NADPH. It was shown that the enzyme undergoes inactivation by oxidation when the level of NADPH is low. From these results, it appears that the regulation of NR in *Chlorella* differs from that in higher plants and *Aspergillus nidulans*. *Sorghum* leaves contain a significant amount of cyanide in the form of a cyanogenic glucoside, dhurrin, in the early stages of development [9]. Recently, regulation of NR by cyanide has been proposed [10]. However, it is not known whether cyanide has any role in the regulation of NR in *Sorghum* leaves. We now show that cyanide-inactivated NR is not present *in vivo* in *Sorghum* leaves.

Contradictory reports are available about the source of reducing power for *in vivo* nitrate reduction in higher plants. It has been proposed that NADH produced during glycolysis is used for nitrate reduction [11]. However, recent studies of Sawhney *et al.* [12] have shown that NADH generated in the reactions subsequent to the triose phosphate dehydrogenase step of glycolysis is also utilized for nitrate reduction in leaves. We now confirm that NADH generated in the citric acid cycle is utilized for *in vivo* nitrate reduction in *Sorghum* leaves.

RESULTS AND DISCUSSION

It is known that during extraction NR from *Sorghum* leaves is inactivated by reaction with HCN, released from the glucoside dhurrin. In order to prevent this, addition of nickel chloride in the extraction buffer is recommended [13]. However, nickel chloride cannot reactivate a pre-existing cyanide-inactivated NR by dissociating the cyanide–enzyme complex. The presence of an inactive NR–cyanide complex in *Sorghum* leaves under physiological conditions is not known. It has been shown that cyanide-inactivated NR from *Chlorella vulgaris* can be reactivated by oxidation with ferricyanide [4]. The results in Table 1 show that the NR extracted in Tris–HCl buffer containing 4 mM nickel chloride is not activated with ferricyanide. On the contrary, higher concentrations of ferricyanide were inhibitory. This indicates that the NR extracted from *Sorghum* leaves does not exist in an inactive form.

It was shown earlier that NR from some higher plants is activated by NADH [6, 7]. NR from *Sorghum* is also activated (25–30%) by pre-incubation with NADH at 0°. Similar results were obtained with a partially purified preparation. Other nucleotides such as NAD, FAD, ADP, NADPH and $FADH_2$ had no effect. Glutathione (reduced) or cysteine in the pre-incubation did not stimulate NR. It has also been shown that pre-incubation of NR from wheat and rice seedlings with NADH at 0° stimulates its activity by enhancing the affinity of NR for nitrate [7].

Table 2 shows the effect of NADH on stability of NR at 30°. The enzyme in the crude extract stored at 30° lost 80% of the activity in 2 hr. Nitrate did not protect the enzyme from inactivation. NADH completely prevented inactivation of enzyme up to 2 hr.

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Table 1. Effect of potassium ferricyanide on nitrate reductase (NR) in *Sorghum* seedlings

Addition to pre-incubation mixture (mM)	NR activity	% control
Nil (control)	4.56	100
$K_3Fe(CN)_6$		
0.30	4.68	103
1.25	4.68	103
1.87	3.96	87
2.50	3.00	66

Enzyme extract was pre-incubated at 0° with potassium ferricyanide for 5 min and assayed as described in Experimental. NR activity was expressed as μmol NO_2^- formed/g fr. wt/30 min.

The enzyme stored at 30° in the presence of NADH had 25–30% more activity than the original. This may be due to activation of the enzyme by NADH.

Table 3 shows that freeze–thaw treatment had no effect on *in vitro* activity of NR. However, *in vivo* NR activity was completely destroyed. A supply of NADH restored the activity of the enzyme. This shows that NADH is a limiting factor for *in vivo* nitrate reduction in such leaves. Heber and Santarius [14] have reported that plant mitochondrial activity is lost by freezing and thawing due to alteration of the lipoproteins in membranes, although activities of individual soluble enzymes are not affected. It is likely that the freeze–thaw treatment causes dislocation of integrated functioning of mitochondria and the citric acid cycle, resulting in loss of NADH generation required for *in vivo* nitrate reduction. In order to confirm the role of the citric acid cycle as a source of reductant, the effect of

Table 2. Time course of inactivation of nitrate reductase (NR) at 30° with and without NADH and KNO_3

Incubation time (min)	NR activity		
	Control	+KNO_3	+NADH
0	100	100	100
30	76	82	127
60	58	45	130
90	33	36	127
120	21	15	124

Crude extract was kept at 30° with and without NADH (0.34 mM) and KNO_3 (10 mM) for varying time intervals and subsequently NR activity was assayed. Activity was compared with control which was taken as 100. Activity in the control was 5.94 μmol NO_2^-/g fr. wt/hr.

malonate, a competitive inhibitor of succinate dehydrogenase, was studied. The inhibitory effect was proportional to the concentration of malonate added. Malonate at 25 mM inhibited nitrate reduction by 95%. When malonate and fumarate were added together, the inhibitory effect of malonate was reversed

Table 3. Effect of freeze–thaw treatment on *in vitro* nitrate reductase (NR) activities in *Sorghum* seedlings

Treatment	NR	
	in vivo	*in vitro*
Control	2.35	10.8
Freezing–thawing	N.D.	10.8
Freezing–thawing 1.25 mM NADH	4.50	10.8

Leaf discs (0.1 g) placed in a thin wall test-tube were immersed in liquid N_2 for 1 min and then thawed at 30°. The freeze–thaw treatment was repeated 5 times. *In vivo* and *in vitro* NR activity was assayed as given in Experimental. NR activity was expressed as μmol NO_2^- formed/g fr. wt/hr.

N.D. = not detectable.

by fumarate to the extent of 60% (Table 4). In order to substantiate these results, the effect of sodium fluoride, in the presence of phosphate, on *in vivo* nitrate reduction was studied. The inhibitory effect was proportional to the concentration of sodium fluoride added. Sodium fluoride at 50 mM inhibited nitrate reduction to the extent of 60%. Sodium fluoride is known to be an inhibitor of enolase and succinate dehydrogenase [15]. Hence, it will prevent the formation of pyruvate through glycolysis and also inhibit the citric acid cycle. Fluoride, however, has no effect on triose phosphate dehydrogenase. These results clearly suggest that NADH generated beyond this step, possibly in the dehydrogenase reactions of citric acid cycle, is utilized for *in vivo* nitrate reduction.

Sorghum leaves contain an appreciable amount of cyanide in the form of cyanogenic glucosides in the early stages of development [9]. Homogenization of the leaves for extraction of NR for *in vitro* assay causes release of HCN from glucosides [13]. The presence of HCN in the cell-free extracts of *Chlorella vulgaris* has been reported [16]. HCN is known to inhibit the *in vitro* activity of NR [17, [8]. Lorimer *et*

Table 4. Reversal of malonate inhibition of nitrate reduction by fumarate

Addition to infiltration medium	μmol NO_2^- formed g fr. wt/hr	Activity % of control
Control (Pi buffer 0.1 M, pH 5)	1.26	100
Malonate (20 mM)	0.09	7
Malonate (20 mM) + fumarate (20 mM)	0.40	32
Malonate (20 mM) + fumarate (40 mM)	0.72	57
Malonate (20 mM) + fumarate (100 mM)	0.79	63

al. [4] detected the presence of a naturally occurring cyanide-inactivated NR in *Chlorella vulgaris*. The inactivated enzyme was reactivated by oxidation with ferricyanide. However, our failure to observe activation of NR from *Sorghum* leaves with ferricyanide suggests that the naturally occurring cyanide-inactivated enzyme may not exist in *Sorghum* leaves, despite the presence of dhurrin.

NR is known to be extremely unstable *in vitro* and subject to a rapid turnover system *in vivo* [1, 17]. The mechanism of inactivation is not known. The presence of NR-inactivating enzymes from the leaves of soybean [19] and rice [20] has been reported. It has been shown that NADH prevents the inactivation of NR by its inactivating enzyme obtained from rice leaves [20]. It is likely that similar type(s) of inactivating factors may be responsible for *in vitro* instability of NR from *Sorghum* leaves.

Klepper *et al.* [11] suggested that, in leaves, NADH for NR is derived from the metabolism of 3-phosphoglyceraldehyde produced during photosynthesis. Recent studies by Mann *et al.* [21] have shown that glycolysis and the pentose phosphate pathway were capable of generating NADH through glyceraldehyde-3-phosphate dehydrogenase. However, the inhibitory effect of malonate on nitrate reduction and its reversal by fumarate, as observed in our studies, further supports the results of Sawhney *et al.* [12] who demonstrated that NADH generated in the reactions subsequent to the triose-phosphate dehydrogenase is also utilized for nitrate reduction in leaves. It is known that plant mitochondria are capable of trans-membrane hydrogen transfer [22]. Thus, NADH generated in the cytoplasm during glycolysis can be oxidized in mitochondria and conversely mitochondrial NADH can be used for nitrate reduction in the cytoplasm.

EXPERIMENTAL

Seedlings of *S. bicolor* (L.) cv Moench CSV-5 were grown with 15 mM KNO_3 in the greenhouse. NR was extracted by macerating the leaves of 10–15 day-old seedlings with 20 vol. of 0.1 M Tris–HCl buffer (pH 8.1) containing 4 mM $NiCl_2$ and 20 mM reduced glutathione according to the method of ref. [13]. The homogenate was centrifuged at 10 000 g for 30 min. The supernatant obtained was used as the source of enzyme.

Partial purification of NR was effected as described in ref. [23] by precipitation with 50% satd $(NH_4)_2SO_4$. The ppt. was redissolved in Pi buffer and passed through a Sephadex G-25 column.

Pre-incubation treatment of the enzyme. The crude extract or partially purified NR was pre-incubated at 0 or 30° with NADH as described in ref. [7]. For subsequent assay, the quantity of NADH added was appropriately adjusted. Nitrate reductase was assayed according to the *in vitro* method of ref. [24]. The reaction mixture contained in μmol: Pi buffer 100, KNO_3 20, NADH 1.25. Nitrite formed after incubation at 30° was determined. Nitrate production *in vitro* was measured in leaf discs after incubating in the dark for 1 hr. Incubation mixture contained: 0.1 M Pi buffer pH 5, 1 ml; *n*-PrOH 0.1 ml; KNO_3 (0.1 M) 0.2 ml and leaf sample 0.1 g. The final vol. was adjusted to 2 ml. Vacuum infiltration was used to facilitate the penetration of metabolites. After 1 hr incubation, the leaves were extracted in H_2O at 100° and nitrite was determined in the extract according to the method of ref. [25].

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