Phytochemistry, Vol. 22, No. 4, pp. 821-824, 1983.
Printed in Great Britain.

0031-9422/83/040821-04$03.00/0

ACTIVATION AND STABILIZATION OF NITRATE REDUCTASE BY NADH IN WHEAT AND MAIZE

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(*Received* 11 *January* 1982)

Key Word Index—*Triticum aestivum*; wheat; *Zea mays*; maize; Gramineae; nitrate reductase; NADH.

Abstract—Preincubation of nitrate reductase (NR) extracted from wheat shoot tips with NADH *in vitro*, activated and stabilized activity at both 0° and 25°. However, preincubation with potassium ferricyanide inactivated the NR *in vitro*. NADH also stabilized the NR activity in extracts from maize shoot tips. It was observed that NR from both wheat and maize was active at low temperatures.

INTRODUCTION

Nitrate reductase (NR) (EC 1.6.6.1) is regulated by its redox potential [1]. In many plants, it exists in two interconvertible forms: an oxidized and active form and a reduced and inactive form [2, 3]. In rice, wheat, barley and *Sorghum*, however, the reduced form is the active form of NR [4–7].

Reduced pyridine nucleotides have been shown to have diverse effects, i.e. inactivation, protection, or activation, on NR extracted from different sources. Inactivation has been reported in *Chlorella* [2, 8–15], *Chlamydomonas reinhardii* [16], *Neurospora crassa* [17], *Nitrobacter agilis* [18], *Ankistrodesmus braunii* [19], spinach [20–22], maize and pea [23], cucumber [24] and rice [25]. Protection has been reported in rice seedlings [26], cotton cotyledons [27], rice cells [28], wheat leaves [29] and corn leaves [30]. Activation has been reported in rice [4, 5, 7] wheat [5, 7], barley [5] and sorghum seedlings [6].

In the present study the effect of NADH and ferricyanide on NR from wheat and maize has been investigated.

RESULTS AND DISCUSSION

To study the effect of NADH on wheat NR, the dialysed tissue extract was preincubated at 0° with 0.5, 1 and 2 mM NADH for 1–2 hr. After preincubation, the NR activity was measured. It was observed that NADH activated NR by *ca* 35% and the maximum activation was obtained at 2 mM NADH after a period of 1 hr (Table 1). This concentration of NADH is higher than that used in earlier studies. Also the preincubation time required for the maximum activation at 0° was found to be longer than indicated in earlier studies.

When the kinetics of activation were followed at 25°, it was observed that within 10 min, the NR activity had increased by 27% (Fig. 1). The maximum activation is, however, achieved in 20 min. This shows that the activation in wheat NR was without a lag phase. By comparison rice NR has a lag phase of 20 min [4]. This suggests that the effect of NADH is direct in the case of

Table 1. *In vitro* effect of different concentrations of NADH and different preincubation times on wheat NR activity

Treatments	Preincubation period	Specific activity	Relative activity
Nitrate reductase		107	100
+ NADH (0.5 mM)	0 hr	106	98
+ NADH (1.0 mM)		109	101
+ NADH (2.0 mM)		106	99
+ NADH (0.5 mM)	1 hr	138	128
+ NADH (1.0 mM)		143	133
+ NADH (2.0 mM)		145	135
+ NADH (0.5 mM)	2 hr	132	123
+ NADH (1.0 mM)		136	126
+ NADH (2.0 mM)		139	129

NR (potassium nitrate induced), extracted as described in the Experimental, was dialysed for 5 hr, after which it was preincubated with different concentrations of NADH at 0° for 1 and 2 hr.

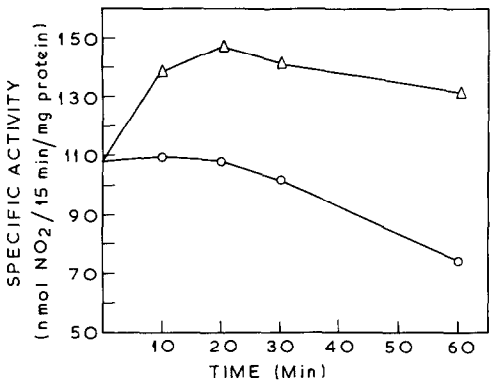

Fig. 1. Initial kinetics of activation of NR by NADH. NR, extracted as described in Experimental was preincubated with 2 mM NADH and the initial kinetics studied at 25° in light. (○) KNO_3 induced enzyme; (△) KNO_3 induced enzyme + NADH.

wheat. It was also seen that NADH increased the half-life of NR at 25° from 1.5 hr to over 4 hr (Fig. 2).

Two hypotheses have been proposed to explain the mechanism of action of NADH. These are: (1) That NR is inactivated by the over reduction of the molybdenum moiety. In cases where cyanide is required for complete inactivation, it may bind to the over-reduced molybdenum moiety to form a complex which is inactive [31]; (2) Besides changing the redox potential, NADH may act by inhibiting the inactivating enzyme [6]. In wheat, it seems that NADH might act by both of these mechanisms. There is also a third possibility, that NADH could bind with NR and change its conformation to a more stable one.

Ferricyanide at 2 mM was found to immediately inactivate NR when preincubated with it at 0° (Table 2). To check whether the 2 mM ferricyanide was interfering with the assay by oxidizing the NADH, the ferricyanide preincubated enzyme was assayed with higher concentrations of NADH. It was found that the decrease in NR activity was not due to lack of NADH in the assay mixture (Table 2).

Inactivation was followed by preincubating NR with 1 mM ferricyanide. It was seen that the inactivation increased with time and a maximum of 49% inactivation was observed after 2 hr (Table 3). When NR, dialysed to remove all of the nitrate, was preincubated with 1 mM ferricyanide, ferricyanide was unable to inactivate the enzyme (Table 3).

Thus, it was seen that ferricyanide inactivated wheat NR and this inactivation required nitrate for its action. Kadam *et al.* [6] reported in *Sorghum*, that ferricyanide had no effect on NR. Nevertheless, where NADH has been reported to inactivate NR, oxidizing agents, such as ferricyanide and nitrate, have reactivated it. In such systems NR seems to exist in two interconvertible forms—one oxidized and active and the other reduced and inactive [2, 32]. In wheat shoot tips, it appears that the reduced form is active and the oxidized form is inactive. As shown by Dunn-Coleman and Pateman [1], the NR of wheat also seems to be regulated by its redox potential. The exact mechanism by which this is brought about and as to why it is the opposite to NR of other plants is unknown.

In maize, NADH stabilized the NR activity. Maize NR has a half-life of 1 hr at 0°. If NADH was added to it, the NR activity was stabilized to a considerable extent and the

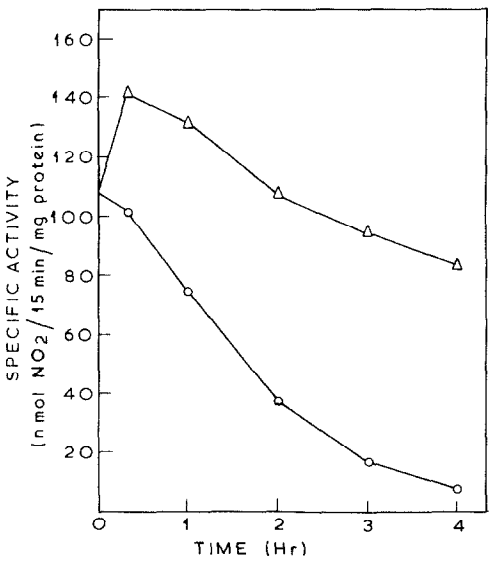

Fig. 2. Kinetics of activation and stabilization of NR by NADH. NR, extracted as described in the Experimental was preincubated with 2 mM NADH and the kinetics studied at 25° in light. (○) KNO_3 induced enzymes; (△) KNO_3 induced enzyme + NADH.

Table 2. Effect of different concentrations of NADH on potassium ferricyanide preincubated wheat NR assay

Treatments	Concentrations of NADH in assay (mM)	Specific activity	Relative activity
Nitrate reductase	1.5	128	100
$+K_3Fe(CN)_6$	1.5	57	44
$+K_3Fe(CN)_6$	3.0	51	40
$+K_3Fe(CN)_6$	4.0	59	46
$+K_3Fe(CN)_6$	6.0	60	47

NR (potassium nitrate induced), extracted as described in the Experimental, was preincubated with 2 mM potassium ferricyanide for 2 hr at 0°, after which it was assayed with different concentrations of NADH.

Table 3. Effect of different preincubation times with potassium ferricyanide on undialysed and dialysed NR from wheat

Treatments	Period of preincubation (hr)	Crude NR		Dialysed NR	
		Specific activity	Relative activity	Specific activity	Relative activity
Nitrate reductase	0	128	100	140	100
$+K_3Fe(CN)_6$	0	106	83	145	105
$+K_3Fe(CN)_6$	0.5	83	65	—	—
$+K_3Fe(CN)_6$	1.0	76	59	148	107
$+K_3Fe(CN)_6$	2.0	66	52	137	99

NR (potassium nitrate induced) was extracted as described in the Experimental. In one experiment NR was dialysed for 2 hr. The crude and dialysed enzymes were preincubated with 1 mM potassium ferricyanide at 0°. The NR activity was estimated at various intervals for 2 hr.

stabilization was greater at 2 mM NADH (Table 4). NR from both wheat and maize is active at zero and sub-zero temperatures (Table 5). NR extracted from wheat and maize shoot tips induced in 0.1 M potassium nitrate when assayed at temperatures varying from $-10°$ to 40° gave the highest activity at 30°. The enzyme was active even at $-10°$. At 0°, the NR activity was *ca* 25% of that at 30°.

Table 4. *In vitro* effect of different concentrations of NADH and different preincubation times on maize NR

Treatments	Preincubation period	Specific activity	Relative activity
Nitrate reductase		178	100
+ NADH (1 mM)	0 hr	181	102
+ NADH (2 mM)	0 hr	171	96
		88	49
+ NADH (1 mM)	1 hr	169	95
+ NADH (2 mM)	1 hr	174	98
		39	22
+ NADH (1 mM)	2 hr	135	76
+ NADH (2 mM)	2 hr	163	92

Nitrate reductase NR (potassium nitrate induced), extracted as described in the Experimental was preincubated with different concentrations of NADH at 0° for 1 and 2 hr.

Table 5. Effect of temperature on the NR assay

Assay temperature (°)	Wheat specific activity	Maize relative activity
−10	11	44
0	28	49
10	84	67
20	121	94
30	124	148
40	23	86

Nitrate reductase was extracted as described in Experimental and assayed at the temperature stated.

EXPERIMENTAL

Etiolated shoot tips of 7-day-old seedlings of *Triticum aestivum* HD2204 and *Zea mays* A 5154 were used. The seeds were germinated in complete darkness at $27 \pm 1°$. For enzyme induction, shoot tips of 5–6 cm length were excised from 7-day-old seedlings and were floated with their basal side down in the induction medium containing 100 mM KNO_3 for wheat and 60 mM KNO_3 for maize, for 18 hr in light (1.2 mW/cm^2). After 18 hr, the enzyme was extracted by homogenizing the shoots in extraction buffer containing 50 mM Pi buffer, pH 7.4, 1 mM EDTA and 1 mM cysteine. The homogenate was centrifuged at 20000 *g* for 20 min. The supernatant was dialysed against the extraction buffer for 5 hr in the cold to remove the nitrate, after which it was preincubated with NADH. The nitrate estimation was done by the method of ref. [33]. For preincubation with ferricyanide, both dialysed and undialysed enzyme were used. The enzyme was assayed according to the procedure of ref. [34], with slight modifications [35]. The protein was assayed according to the method of ref. [36].

In maize, the same procedure was followed, except that the extraction buffer consisted of 50 mM Tris buffer containing 3 mM EDTA and 3 mM cysteine. Also preincubations were done without dialysing the enzyme, since the maize NR is very labile.

All treatments were given in duplicate and each expt is repeated at least twice. The sp. act. is expressed as nmol NO_2^-/15 min/mg protein.

Acknowledgement—N. D. and L.V.M.R. are grateful to the C.S.I.R. for their Senior Research Fellowships.

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