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Communication

Effect of Inorganic Orthophosphate on *in Vitro* Activity of NADH-Nitrate Reductase Isolated from 2-Row Barley Leaves

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ABSTRACT

Inorganic orthophosphate (25 millimolar in assay media; Pi) was found to increase *in vitro* activity of NADH-nitrate reductase (NR) isolated from 2-row barley (*Hordeum vulgare* L.) leaves with a saturating concentration of nitrate (2 millimolar) but to decrease it with low nitrate levels (<0.1 millimolar). The response to nitrate concentrations was Pi specific. The Lineweaver-Burk plot showed that Pi increases the apparent K_m for nitrate as well as V_{max}, whereas it does not alter the K_m for NADH significantly. These results suggest that the interaction between a molybdenum site of the enzyme and Pi results in alteration of the properties of NR molecule.

The enzyme NADH-NR[1] (EC 1.6.6.1) catalyzes the reduction of nitrate to nitrite by NADH. It constitutes the first step in the assimilatory reduction of nitrate to ammonia in the tissues of most plants. It is known that Pi activates NR (5–7, 12), and it is customary to assay the enzyme in Pi buffer at a final concentration between 25 and 100 mM (2, 3). The mechanism and kinetic features of the activation have not been completely carried out, but the major effect seems to be on V_{max} rather than on substrate binding (5). It has been suggested that Pi binds at or near the molybdenum cofactor of NR molecule, possibly resulting in an enhanced potential for reducing nitrate to nitrite (5, 6). There are no extensive studies on Pi requirement of higher plant NR. In a previous paper (8), the effect of various anions on the *in vitro* NADH-NR activity was tested, and Pi (10 mM) activated it by a factor of 1.5. However, this effect was not further analyzed. In this report we describe two different effects on *in vitro* activity of NADH-NR caused by its interaction with Pi.

MATERIALS AND METHODS

Plant Material. Two-row barley seeds (*Hordeum vulgare* L. cv Daisen-gold) were germinated at 20°C in the dark for 3 d, after which they were transferred to an environmentally controlled room (10) with a 12/12-h day/night regime of 25°C and 20°C, respectively, and grown on tap water for 2 d. After further 4-d culture on the nutrient medium described by Oji *et al.* (9), the leaves were harvested then frozen in liquid N_2 and stored at −25°C until used.

NR Preparation. NR was purified from the frozen leaves as reported previously (8), except that the final purification step was omitted. In short, the procedure involved $(NH_4)_2SO_4$ fractionation (25–45% saturation) from the crude extract, adsorption on Phenyl Sepharose CL-4B and hydroxyapatite, and followed by affinity chromatography on Blue Sepharose CL-6B.

Pi-free NR was prepared from the affinity-purified enzyme by passage through the Bio-Gel P-6DG column (1.5 × 15 cm) equilibrated with 25 mM Hepes-NaOH, pH 7.5. The enzyme had a specific activity of 14 μmol nitrite formed min^{-1} mg^{-1} protein under optimal conditions. The enzyme was stored at −25°C until used.

NADH-NR Assay. Unless otherwise stated, the reaction mixture having a final volume of 2 ml contained: 50 μmol Hepes-NaOH (pH 7.5), 4 μmol KNO_3, 0.66 μmol NADH, 0.002 μmol FAD, and NR preparation (0.3–0.5 μg protein). The nitrite formed was determined as described previously (9).

MVH-NR Assay. The reaction mixture contained 50 μmol Hepes-NaOH (pH 7.5), 1.33 μmol MV 1.6 to 20 μmol KNO_3, and enzyme in a total volume of 1.9 ml. The reaction was started by adding 0.1 ml of a freshly prepared solution of 10 mg/ml $Na_2S_2O_4$ dissolved in 100 mM $NaHCO_3$. After 10-min incubation at 30°C the reaction was terminated by vortexing which oxidized the MVH and any remaining dithionite. Nitrite was estimated according to the procedure described for NADH-NR with post-assay treatments as described by Senn *et al.* (13).

NADH-CR Assay. The reaction mixture contained 75 μmol Hepes-NaOH (pH 7.5), 1.5 mg Cyt *c* (horse heart type III, Sigma), 0.66 μmol NADH, and enzyme in a total volume of 3 ml. The reaction was followed at 25°C by measuring the *A* change at 550 nm. The activity was also assayed in the presence of varying nitrate concentrations (0.05–2.0 mM). Added nitrate did not cause detectable inhibition of NADH-CR component of the enzyme as was observed by Solomonson and Vennesland (14).

All assays were run in duplicate.

RESULTS AND DISCUSSION

The effect of Pi on *in vitro* NADH-NR activity of Pi-free purified enzyme, in the presence of various buffers, is shown in Table I. The assays were carried out at 2 mM KNO_3. Pi buffer gave the maximal activity. The addition of Pi to the non-Pi buffers resulted in an almost complete restoration of the rate obtained in Pi alone. K- and Na-Pi behaved identically. The activity was measured as a function of Pi concentration at 2 mM KNO_3 (Fig. 1). The activity increased markedly as the Pi concentration was increased from 0 to 20 mM and decreased sharply above 60 mM Pi. Between 20 and 50 mM Pi, it remained constant. Half-maximal activation occurred around 4 mM Pi at pH 7.5. A similar response was reported by Renosto *et al.* (11) with *Peni-*

[1] Abbreviations: NR, nitrate reductase; MV, methyl viologen; MVH, reduced methyl viologen; CR, cytochrome *c* reductase.

Table I. *Effect of Pi on in Vitro NADH-NR Activity with 2 mM KNO_3 in the Presence of Various Buffers*

The pH of each buffer was 7.5.

Buffer Used (25 mM)	Pi (25 mM)	NADH-NR Activity	
		nmol·min⁻¹	*relative*
Hepes-NaOH		4.47	100
Tris-Cl		4.60	103
Tricine-NaOH		5.14	115
K-Pi		6.79	152
Na-Pi		6.61	148
Hepes-NaOH	K-Pi	6.62	148
Hepes-NaOH	Na-Pi	6.81	152
Tris-Cl	K-Pi	6.57	147
Tricine-NaOH	K-Pi	6.84	153

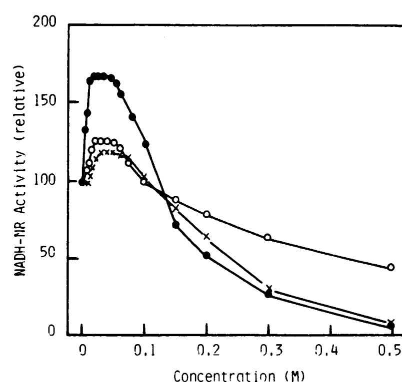

FIG. 1. The effect of Pi (●), sulfate (○), and chloride (×) on *in vitro* NADH-NR activity with 2 mM KNO_3. Pi was prepared by mixing equimolar solution of KH_2PO_4 and K_2HPO_4 until the pH was 7.5. Sulfate and chloride was used as sodium salts.

Table II. *Nitrate Concentration-Dependent Variations in Pi Effect on in Vitro NADH-NR Activity*

Pi was prepared by mixing equimolar solutions of KH_2PO_4 and K_2HPO_4 until the pH was 7.5.

Assay System		NADH-NR Activity	
Nitrate	Pi		
mM	*mM*	*nmol·min⁻¹*	*relative*
0.05		2.42	100
0.05	5	2.09	86
0.05	25	1.51	62
0.2		3.30	100
0.2	5	4.32	131
0.2	25	3.76	114
2.0		3.91	100
2.0	5	5.66	145
2.0	25	6.63	170

cillium NADPH-NR.

The effect noted above were not Pi specific. Chloride and sulfate also increased the activity (Fig. 1). However, the maximal activation was only about 20 to 40% of that observed with Pi. Like Pi, a variety of anions (chloride, sulfate, nitrate) inhibited at high concentrations, suggesting that the activity is sensitive to ionic strength changes.

When assays were performed under conditions in which the nitrate concentration is 0.05 mM, 25 mM Pi decreased the activity (Table II). Further, the optimal Pi concentration for the reaction was shifted from 25 mM with 2 mM KNO_3 to 5 mM with 0.2 mM. These effects were Pi specific. Neither chloride nor sulfate at the corresponding concentration decreased the NADH-NR activity measured at 0.05 mM KNO_3 (data not shown). PPi was as effective as Pi with 0.05 mM KNO_3 but not with 2 mM KNO_3 (Table III).

Table III. *Effect of Inorganic Pyrophosphate on in Vitro NADH-NR Activity with 0.05 and 2.0 mM KNO_3*

The pyrophosphate was used as a sodium salt adjusted to pH 7.5 with HCl.

Assay System		NADH-NR Activity	
Nitrate	PPi (25 mM)		
mM		*nmol·min⁻¹*	*relative*
0.05	−	2.52	100
0.05	+	1.49	59
2.0	−	3.93	100
2.0	+	4.52	115

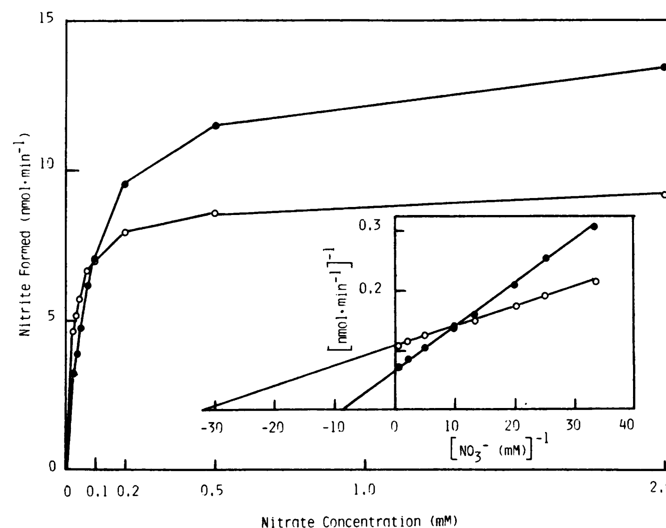

FIG. 2. The effect of Pi on NADH-NR activity with nitrate as variable substrate. Inset: Lineweaver-Burk plots. (○), no Pi. (●), plus 25 mM Pi (pH 7.5).

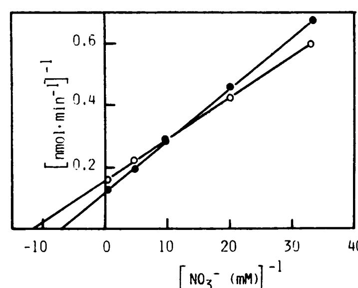

FIG. 3. The effect of Pi on NADH-NR activity with nitrate as variable substrate in the presence of 40 mM NaCl. (○), no Pi. (●), plus 25 mM Pi (pH 7.5).

The apparent K_m for nitrate was determined with and without Pi (Fig. 2). Lineweaver-Burk plots were linear both in the presence and absence of Pi, indicating that there was no evidence for cooperativity in substrate binding; Pi increased the K_m for nitrate as well as V_{max}. The K_m values were 120 μM and 31 μM, respectively, with and without 25 mM Pi. Figure 2 indicates that two lines intersect at the concentration corresponding to about 0.1 mM nitrate. Another experiment with 5 mM Pi showed 68 μM for the nitrate K_m. Pi did not alter the K_m for NADH significantly (data not shown).

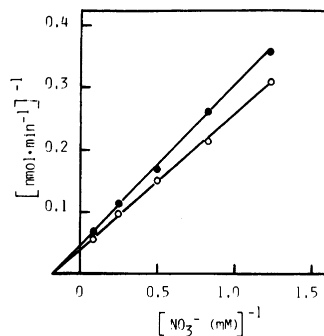

FIG. 4. The effect of Pi on MVH-NR activity as a function of nitrate concentration. (○), no Pi. (●), plus 25 mM Pi (pH 7.5).

These results are contrary to those of Howard and Solomonson (5) who indicated that Pi did not alter the nitrate K_m with *Chlorella* NADH-NR. Thus, we examined the Pi effect under the conditions used by them (with 40 mM NaCl). A similar tendency was also obtained in the presence of NaCl (Fig. 3), though the Pi effect was a lesser extent. The K_m values for nitrate were 140 μM and 90 μM, respectively, with and without 25 mM Pi. NaCl (40 mM) also increases the nitrate K_m, suggesting that ionic strength changes affect the nitrate K_m of the enzyme.

It is of interest to examine how Pi and nitrate interact on the NADH-CR and MVH-NR partial reactions. Pi (25 mM) had no significant effect on NADH-CR activity associated with NR (data not shown). Pi slightly inhibited MVH-NR activity. No interaction of Pi and nitrate was observed (Fig. 4). The K_m for nitrate was a very high value (5.8 mM).

The variations of the nitrate K_m values (31 to 5800 μM) suggest that optimal orientation of nitrate by the enzyme is important in the formation of the nitrate-molybdenum complex. Some NRs are reported to show two K_m values (1). Hewitt and Notton (4) have tentatively explained this phenomenon by supposing that nitrate forms a ligand with Mo^{5+} (small K_m) more readily than with Mo^{6+} (large K_m) and that these alternatives are directly related to the reaction kinetics of the enzyme. They also suppose that Mo^{5+} may be further reduced to Mo^{4+} and that unliganded Mo^{4+} state reacts slowly with nitrate. We feel that the alteration of the nitrate K_m may result from the difference in participant species of molybdenum.

It is difficult to explain the interaction of Pi and nitrate (Figs. 2 and 3) on the basis of an ionic strength change only. Perhaps Pi causes a specific and nonspecific binding to the molybdenum site on the enzyme and thereby affects its affinity for the nitrate. Nevertheless, the interaction between the molybdenum site and added Pi may result in alteration of the properties of NR molecule.

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