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NAD(P)H BISPECIFIC NITRATE REDUCTASE IN BARLEY LEAVES: PARTIAL PURIFICATION AND CHARACTERIZATION*

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; kinetics; nitrate reductase; NAD(P)H nitrate reductase.

Abstract—A nitrate reductase was isolated from a barley (*Hordeum vulgare* L.) mutant (*nar* 1a) deficient in NADH nitrate reductase (EC 1.6.6.1). The nitrate reductase from *nar* 1a exhibited activity with both NADH and NADPH as electron donors. The enzyme was purified 200 fold with a recovery of 49%. Based upon kinetic, catalytic, and immunological characteristics the enzyme was determined to be a NAD(P)H bispecific nitrate reductase (EC 1.6.6.2) with an NADPH to NADH activity ratio of 1.8. The bispecific enzyme cross-reacted with antiserum raised against the NADH nitrate reductase from the wild type but with a much lower specificity than the NADH enzyme. The K_m for nitrate of the NAD(P)H enzyme was 0.61 mM which was about five times greater than that of the NADH enzyme. The K_m for NADPH and NADH of the bispecific enzyme were 10 μM and 68 μM, respectively, while the K_m for NADH of the NADH enzyme was 10 μM. Unlike the NADH enzyme, the NAD(P)H bispecific enzyme was inhibited by dithionite and NADPH. These data support the conclusion that the nitrate reductase in the *nar* 1a mutant of barley is a single NAD(P)H bispecific enzyme.

INTRODUCTION

Nitrate reductase in barley (*Hordeum vulgare* L.) has many characteristics in common with nitrate reductases from other species. Purified barley nitrate reductase is capable of nitrate reduction with NADH, $FMNH_2$ and reduced methyl viologen as electron donors, and has cytochrome *c* reductase activity [1]. Most higher plant nitrate reductases are specific for NADH (EC 1.6.6.1) although NAD(P)H bispecific nitrate reductases (EC 1.6.6.2) have been reported and characterized from several species [2–6].

Ten barley nitrate reductase-deficient mutants have been isolated and characterized in our laboratory [7, 8]. Nine of these are allelic and are believed to be nitrate reductase structural gene mutants (*nar* 1). The tenth has been identified as a molybdenum cofactor mutant because it lacks both nitrate reductase and xanthine dehydrogenase activities and is able to complement *in vitro* with the molybdenum cofactor from xanthine oxidase [8–10]. All of the *nar* 1 mutants have low levels of nitrate reductase activity and are capable of substantial growth with nitrate as a sole nitrogen source [11]. Based upon pH optimum, cofactor preference and substrate kinetics, Dailey *et al.* [12] concluded that the low level of nitrate reductase activity in *nar* 1a (formerly Az12) was due to a NAD(P)H bispecific nitrate reductase and was not the result of a leaky mutation in the NADH nitrate reductase gene. These studies were conducted with ammonium sulphate precipitated preparations and attempts to purify the enzyme from *nar* 1a were unsuccessful.

The objectives of this study were to purify the NAD(P)H bispecific nitrate reductase from mutant *nar* 1a, to characterize the enzyme, and to compare it with the NADH nitrate reductase from the wild type.

RESULTS

The nitrate reductase from *nar* 1a which exhibits activity with both NADH and NADPH was isolated by a procedure similar to that used by Kuo *et al.* [1, 13] to purify the barley NADH enzyme. The enzyme was purified *ca* 200 fold with a recovery of 49% (Table 1). The initial specific activity was only 12% of that reported for the barley NADH nitrate reductase [1]. Although recoveries were comparable (50%), final specific activities were only 5% of those previously reported [1] for the barley NADH nitrate reductase. This relative decrease was perhaps a function of the 10-fold larger scale purification used to isolate the enzyme from *nar* 1a and the associated difficulties in removing casein and bovine serum albumin which were necessary to minimize the effects of plant phenolics and proteases [1, 14]. Nitrate reductase from *nar* 1a was sensitive to precipitation by ammonium sulphate. Dry ammonium sulphate precipitation, as used with the NADH enzyme [1], resulted in recoveries of less than 30% after Sephadex G-25 chromatography. Precipitation with equal volumes of saturated ammonium sulphate solutions more than doubled recoveries (Table 1).

Previous attempts to purify nitrate reductase from *nar* 1a were hampered by poor binding to Blue Dextran

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Table 1. Purification summary of nitrate reductase isolated from *nar* 1a mutant of barley

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)	NADPH NADH
Homogenate	9.7	5560	0.002	1	100	1.7
0–50% $(NH_4)_2SO_4$*	—	—	—	—	—	1.8
Sephadex G-25	6.7	479	0.014	7	69	1.6
Blue A Sepharose	4.8	11.3	0.428	214	49	1.8

*The NAD(P)H enzyme is reversibly inhibited by high salt concentrations rendering measurements of activity at this step spuriously low (yield 17%).

Procedures are described in the Experimental.

Sepharose and AffiGel Blue [12]. The enzyme bound well to the Blue A Sepharose column as shown by negligible activity in the column effluent prior to elution, and by good recovery (Table 1). The enzyme was stable for several months when stored in liquid nitrogen. At room temperature in the air the NADH and NADPH activities of the enzyme both had a half-life of 1.5 hr. Incubation at lower temperatures or under N_2 increased *in vitro* stability (data not shown).

The nitrate reductase had $FMNH_2$ and reduced methyl viologen nitrate reductase activities (Fig. 1A). However these activities were difficult to estimate because dithionite, which was used to reduce methyl viologen and FMN, inhibited the enzyme. With NADH or NADPH as the electron donor, the enzyme was completely inhibited by dithionite concentrations which had little or no effect upon the NADH enzyme (Fig. 1B). The reduced methyl viologen and $FMNH_2$ activities were inhibited by dithionite concentrations greater than 1.5 mM, although published procedures [15, 16] recommend dithionite concentrations of 3 mM. The procedure described by Amy and Garrett [17] for detecting reduced methyl viologen nitrate reductase activity on polyacrylamide gels has been used to identify the barley NADH enzyme [1, 9] but could not be used to detect the enzyme from *nar* 1a, possibly due to inhibition by dithionite.

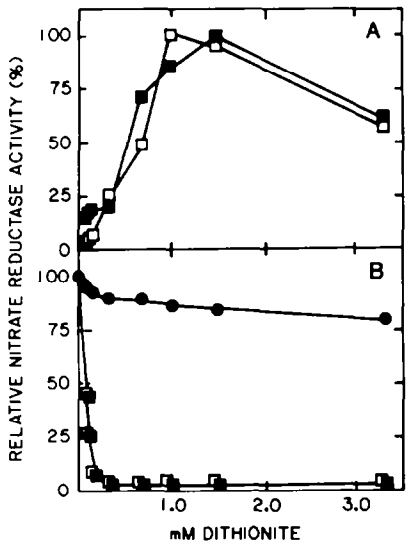

Fig. 1. Influence of dithionite on partially purified barley nitrate reductase. A, NAD(P)H enzyme assayed with reduced methyl viologen (□) or $FMNH_2$ (■) as the electron donor. B, NADH enzyme with NADH as the electron donor (●); NAD(P)H enzyme with NADPH (■) or NADH (□) as the electron donor.

Although the nitrate reductase isolated from *nar* 1a exhibited activity with both NADH and NADPH as electron donors, the evidence indicated that both activities are properties of a single NAD(P)H bispecific enzyme. The NAD(P)H bispecific nitrate reductase had identical pH optima for the NADH and NADPH activities (Table 2). The ratio of the two activities was constant during isolation (Table 1) and both activities were eluted together from the Blue A column by NADPH. The NADH and NADPH nitrate reductase activities of the bispecific enzyme were usually enhanced 5–35% by FAD in the assay medium (data not shown). The NADH enzyme did not respond to FAD. The NAD(P)H bispecific nitrate reductase also exhibited cytochrome *c* reductase activity. The ratio of NADPH/NADH cytochrome *c* reductase activity (2.0) was similar to the ratio of nitrate reductase activity (Table 1). Both the NADH and NADPH enzyme activities decayed at the same rate when stored at room temperature (date not shown) and were inactivated in a similar manner by antiserum developed against the NADH nitrate reductase (Fig. 2).

The NADH enzyme from the wild type was inactivated by much smaller quantities of the antiserum than either the NADH or the NADPH activities of the enzyme from *nar* 1a. Although the NAD(P)H bispecific nitrate reductase cross-reacted with and was inactivated by antiserum to the

Table 2. Characteristics of the NAD(P)H bispecific nitrate reductase from *nar* 1a mutant of barley as compared with the NADH nitrate reductase from the wild type

	nar 1a		Wild type*
Property	NADPH	NADH	NADH
pH optimum	7.7	7.7	7.5
Temperature optimum	31	31	—
K_m NO_3^- (mM)	0.61	0.62	0.13
K_m NAD(P)H (μM)	10	68	10

*See ref. [12].

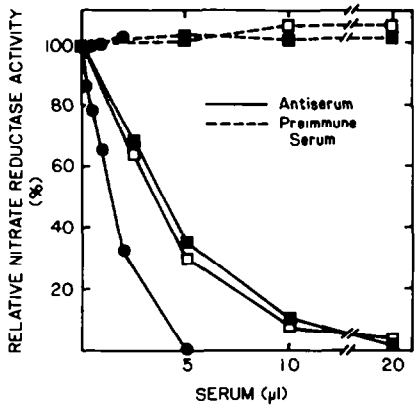

Fig. 2. Inhibition of partially purified barley nitrate reductases by antiserum developed against the NADH enzyme. NADH enzyme with NADH as the electron donor (●); NAD(P)H bispecific enzyme with NADPH (■) or NADH (□) as the electron donor. All samples were incubated with antiserum (—) or preimmune serum (- - -) for 1 hr at room temperature before assay initiation by addition of the pyridine nucleotide. Assays were conducted for 30 min in a volume of 0.5 ml. Control activities were 44 nmol NO_2^-/30 min for the NADH enzyme, and 22 nmol NO_2^-/30 min (NADPH) and 12 nmol NO_2^-/30 min (NADH) for the NAD(P)H bispecific enzyme.

NADH enzyme, it did not form a detectable immunoprecipitate by either line or rocket immuno-electrophoresis. It was possible, however, to detect the NAD(P)H nitrate reductase polypeptide on immunoblots after separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The NAD(P)H nitrate reductase migrated slightly faster than the NADH enzyme and had a subunit M_r of *ca* 100 000 compared with an M_r of about 110 000 for the NADH enzyme.

The kinetic properties of the nitrate reductase from *nar* la also indicated that the NADH and NADPH activities were properties of a single NAD(P)H bispecific enzyme. The apparent K_m for nitrate was 0.62 and 0.61 mM with NADH and NADPH, respectively, as the electron donor (Fig. 3A). These values are about five times greater than the NADH enzyme K_m for nitrate [12]. A major difference between the NADH and NADPH activities of the enzyme from *nar* la was observed in response to NAD(P)H concentrations. The apparent K_m for NADH was 68 μM. The K_m for NADPH was difficult to estimate by double reciprocal plots due to substrate inhibition at NADPH concentrations greater than 50 μM (Fig. 3B) and insufficient nitrite production for reliable detection at NADPH concentrations below 10 μM. The Halwachs [18] method which is especially useful when substrate conversion is too high to obtain a straight line in the substrate concentration time course, was more reliable and gave an apparent K_m for NADPH of 10 μM (Table 2, Fig. 3C). This is similar to the NADH K_m reported for the barley NADH nitrate reductase [12].

The substrate inhibition by NADPH appears to be an intrinsic property of the NAD(P)H bispecific enzyme and not due to contaminants in the NADPH. NADPH purified by reverse-phase high pressure liquid chromatography [19] produced results identical to those in Fig. 3B (data not shown). Furthermore the inhibition by NADPH

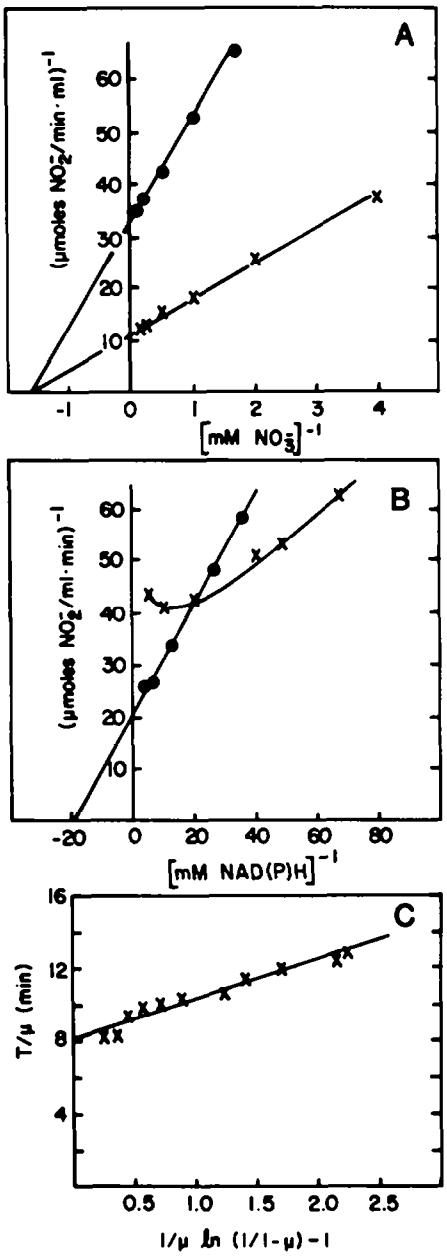

Fig. 3. Kinetic analysis of partially purified nitrate reductase from *nar* la with NADPH (×) or NADH (●) as the electron donor. A, Nitrate reductase activity as a function of nitrate concentration. Pyridine nucleotide concentration was 100 μM. B, Nitrate reductase activity as a function of pyridine nucleotide concentration. Nitrate concentration was 10 mM. C, Halwachs [21] plot of NADPH nitrate reductase activity. T = time in min; μ = fraction of NADPH used at time T; K_m = [NADPH] initial × slope/(intercept—slope).

was reversible. The rate of NADPH oxidation increased as NADPH concentration decreased (Fig. 4). The maximum rate of NADPH oxidation occurred over the NADPH concentration range of 50–150 μM. NADH also exhibited substrate inhibition but only at concentrations greater than 200 μM (Fig. 4).

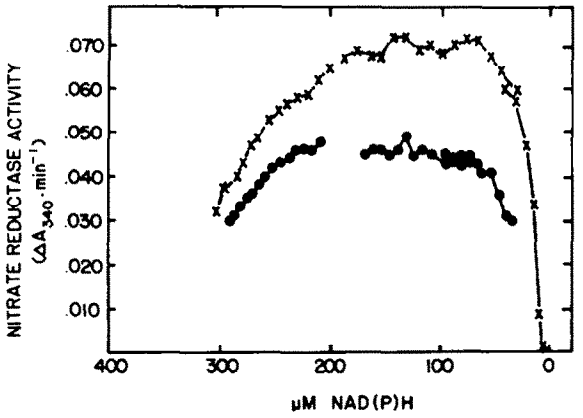

Fig. 4. Activity of partially purified nitrate reductase from *nar* 1a as a function of NADPH (×) or NADH (●) concentration. Enzyme activity and pyridine nucleotide concentration were determined by measuring A_{340} at 1 min intervals. A molar extinction coefficient of 6.2×10^3 was used in calculations of both NADPH and NADH concentrations. Assays were split into three 15 min segments to minimize effects of enzyme decay. Assays were run in a Gilford spectrophotometer at *ca* 25°.

DISCUSSION

Barley mutants with a defective NADH nitrate reductase structural gene (*nar* 1) have low NAD(P)H nitrate reductase activities [12]. The characteristics of the NADH and NADPH nitrate reductase activities from *nar* 1a were similar and appeared to be properties of a single NAD(P)H bispecific enzyme. The two activities remained together during elution from the Blue A column by NADPH (Table 1). They had identical pH and temperature optima, had the same half-life at room temperature, and were inactivated in a similar manner by antiserum raised against the NADH nitrate reductase from the wild type (Table 2; Fig. 2). Furthermore the K_m values for nitrate were the same with either NADH or NADPH as the electron donor (Fig. 3B). Only the K_m values for NADH and NADPH were significantly different (Table 2). Collectively these data indicate that the NAD(P)H nitrate reductase activities in *nar* 1a are due to a single NAD(P)H bispecific nitrate reductase.

NAD(P)H bispecific nitrate reductases from barley and several other higher plant species have characteristics in common that distinguish them from NADH nitrate reductases. The bispecific nitrate reductases can use either NADH or NADPH for nitrate reduction, but activities are greater with NADPH than NADH [4, 5, 12]. The NADPH/NADH ratio of the enzyme from *nar* 1a has been reported to be variable [12] but this is probably due to disparate K_m values for the two dinucleotides and the problems associated with the NADPH substrate inhibition. When the two dinucleotides were prepared at 100 μM and monitored spectrophotometrically, the NADPH/NADH nitrate reductase activity ratio was *ca* 1.8:1. Ratios of *ca* 1.1:1, 1.5:1 and 2:1 have been reported for bispecific nitrate reductases from *Erythrina senegalensis* [6], soybean [4] and rice [5], respectively. In addition the pH optima for the NADH and NADPH activities are the same for NAD(P)H bispecific nitrate reductases [4, 6, 12] but differ for NADH enzymes [4, 12].

In these studies the NAD(P)H bispecific enzyme was immunologically different from the barley NADH nitrate reductase (Fig. 2). At high serum concentrations (0.2%) the cross-reaction between the NAD(P)H enzyme and the NADH enzyme antiserum was sufficient to inhibit the enzymatic activities but not sufficient to form an immunoprecipitate. This antiserum readily inhibited and formed an immunoprecipitate against barley NADH nitrate reductase [9, 20] as well as wheat NADH nitrate reductase [21]. The antiserum raised against the NADH nitrate reductase cross-reacted with nitrate reductases from a wide range of species but formed an immunoprecipitate only with the enzymes from closely related cereal species [21]. On this basis, the NAD(P)H bispecific nitrate reductase from the *nar* 1a mutant of barley would appear to be rather distantly related to the NADH nitrate reductase from the wild type. The NAD(P)H bispecific enzyme had a slightly smaller subunit M_r than the barley NADH enzyme which could contribute to the immunological differences. However major differences in antigenic sites of these enzymes probably exist.

Some kinetic properties of the enzyme from *nar* 1a are also characteristic of NAD(P)H bispecific nitrate reductases. The apparent K_m values for nitrate are similar with NADH or NADPH as the electron donor, but are greater than the K_m for the NADH enzyme. Typically, K_m values of 0.1–0.3 mM nitrate have been reported for NADH nitrate reductases and values 10–50 times greater for the NAD(P)H bispecific enzymes [4, 6, 12]. In barley the difference in K_m values for the two enzymes is at least five fold [0.13 mM for the NADH enzyme and 0.62 for the NAD(P)H enzyme].

The barley NAD(P)H bispecific nitrate reductase differs somewhat from the bispecific enzyme from other species in that the apparent K_m values for NADH and NADPH are different, and NADPH exhibits substrate inhibition. The significance of these observations is not readily apparent but could relate to the *in vivo* importance of the enzyme in the respective organisms. The NAD(P)H bispecific enzyme is presumably very important in *E. senegalensis* where it seems to be the only nitrate reductase present [6]. In soybean the bispecific enzyme represents a significant portion of the total nitrate reductase activity [4]. In barley however, the NAD(P)H enzyme does not appear to be expressed in leaves of the wild type, and is present in only small quantities in *nar* 1 mutants [12, 22]. The NAD(P)H bispecific nitrate reductase may, however, play a significant role in nitrate reduction in *nar* 1 mutants.

EXPERIMENTAL

Plant growth. Seedlings were grown in vermiculite for 8 days in a growth chamber at 16° under continuous illumination (300 $\mu E/m^2/sec$). The plants were watered by subirrigation with a nitrogen-free nutrient soln [23]. Nitrate reductase was induced by watering with the same nutrient soln containing 15 mM nitrate at 24 and 12 hr prior to harvest.

Enzyme extraction. Following the 24 hr nitrate induction period, shoots were excised above the coleoptile, weighed and frozen in liquid N_2. The total shoot mass (325–375 g) was ground in liquid N_2 using a Waring blender. The plant powder was extracted with 3 ml/g fr. wt of 250 mM Tris–HCl (pH 8.2), 1 mM EDTA, 3 mM dithiothreitol, 5 μM Na_2MoO_4, 5 μM FAD, 100 μM phenylmethylsulphonyl fluoride, 1 μg/ml pepstatin, 5 μM antipain and 0.75% casein. The homogenate was centrifuged at 27000 *g* for 1 hr. The supernatant was filtered through

two layers of Miracloth and served as the crude homogenate. All procedures were conducted at 2–4° unless otherwise indicated.

Protein purification. The crude homogenate was immediately mixed with an equal vol. of $(NH_4)_2SO_4$ satd 25 mM Tris buffer (pH 8.2). The mixture was allowed to stand for 30 min and was then centrifuged at 27000 *g* for 10 min at 2–4°. The pellets were dissolved in one tenth the vol. of crude homogenate with 25 mM Tris-HCl (pH 8.2), 1 mM EDTA, 1 μM Na_2MoO_4, 5 μM antipain, 1 μM FAD, and 1 mM dithiothreitol (buffer A). The suspension was centrifuged at 27000 *g* for 30 min at 2–4°, decanted through two layers of Miracloth, and then desalted on a Sephadex G-25 column equilibrated with buffer A. A fraction equal to twice the loaded vol. was collected after the sample front and applied to a Blue A Sepharose (Amicon) column equilibrated with buffer A containing 10 μM Na_2MoO_4 and 10 μM FAD. The column was washed for 18 hr with the same buffer containing 10 μg/ml bovine serum albumin (BSA). Nitrate reductase was eluted with a 0–50 μM NADPH gradient (150 ml total vol.) in 10 μM Tris-HCl (pH 8.2), 1 mM EDTA, 1 μM Na_2MoO_4, 1 μM FAD, and 1 mM dithiothreitol. KNO_3 was added to each fraction (final concn 1 mM) to maintain the enzyme in an oxidized form. Each fraction was assayed for NAD(P)H nitrate reductase activity [12]. The peak fractions (20–25 ml) were pooled and concd to *ca* 4 ml in an Amicon ultrafiltration cell (XM50 membrane).

Electrophoresis. SDS-PAGE was performed according to ref. [24]. Slab gels were 10% acrylamide, 0.1% bisacrylamide and 1.5 mm thick. Samples were adjusted to 4% SDS, 5% β-mercaptoethanol and 10% glycerol and boiled for 2 min. Nitrate reductase on gels was visualized by a nitrate reductase specific immunoblot technique (western blot) [9, 25]. The gels were blotted for 2 hr to nitrocellulose sheets (Millipore Corporation, 0.45 μM) in a Hoeffer transblotter fitted with cooling coils (4°). The blotted sheets were incubated 18 hr at room temp. in 50 mM NaPi (pH 7.4) containing saline (0.1% NaCl) and 1% BSA. Monospecific antiserum raised against the NADH nitrate reductase [9] was mixed in NaPi buffered saline (1:500 dilution) containing 0.5% BSA, 0.55% Tween 20 and 3% PEG-4000. The nitrocellulose sheets were incubated in this mixture at room temp. for 8 hr, washed vigorously for 6 hr with NaPi buffered saline containing 0.05% Tween 20, and incubated for 3–4 hr at room temp. with peroxidase-conjugated antirabbit goat serum diluted (1:1000) in NaPi buffered saline containing 0.5% BSA and 0.05% Tween 20. The sheets were then successively washed in cold (0–2°) NaPi buffered saline containing 0.05% Tween 20 (45 min), NaPi buffered saline (10 min), and 50 mM NaPi buffer pH 7.4 (10 min). Nitrate reductase was visualized by staining the sheets for peroxidase activity for 15–30 min. The blots were then washed in H_2O, dried and stored in the dark.

Assays. Nitrate reductase was assayed as previously described using NAD(P)H [12], reduced methyl viologen [12, 15] or $FMNH_2$ [16], except FAD (1 μM) was included in the NAD(P)H assay medium. One unit of activity is defined as 1 μmol NO_2^- produced per min at 30°. Cytochrome *c* reductase was assayed as described in ref. [26]. Protein was estimated according to ref. [27] using BSA as the standard. Estimates of nitrate reductase specific activity in crude extracts were based upon the plant protein and not the exogenous protein included in the extraction buffer to protect the enzyme. All other specific activities were based upon total protein in the fraction. In some experiments, NADPH purified by reverse-phase high pressure liquid chromatography [19] was used to eliminate low levels of contaminants present in commercial NADPH. The purified NADPH was a gift of Dr. R. G. Yount, Washington State University, Pullman, WA.

Kinetics. Kinetics studies were performed on the Blue A particularly purified nitrate reductase after the preparation was passed through a G-25 column to remove nitrate. K_m values for NADPH were determined by the method of ref. [18]. All other K_m values were determined from Lineweaver-Burk plots.

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