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Characteristics of a Nitrate Reductase in a Barley Mutant Deficient in NADH Nitrate Reductase[1]

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ABSTRACT

A barley (*Hordeum vulgare* L.) mutant, *nar*1a (formerly Az12), deficient in NADH nitrate reductase activity is, nevertheless, capable of growth with nitrate as the sole nitrogen source. In an attempt to identify the mechanism(s) of nitrate reduction in the mutant, nitrate reductase from *nar*1a was characterized to determine whether the residual activity is due to a leaky mutation or to the presence of a second nitrate reductase. The results obtained indicate that the nitrate reductase in *nar*1a differs from the wild-type enzyme in several important aspects. The pH optima for both the NADH and the NADPH nitrate reductase activities from *nar*1a were approximately pH 7.7, which is slightly greater than the pH 7.5 optimum for the NADH activity and considerably greater than the pH 6.0 to 6.5 optimum for the NADPH activity of the wild-type enzyme. The nitrate reductase from *nar*1a exhibits greater NADPH than NADH activity and has apparent K_m values for nitrate and NADH that are approximately 10 times greater than those of the wild-type enzyme. The *nar*1a nitrate reductase has apparent K_m values of 170 micromolar for NADPH and 110 micromolar for NADH. NADPH, but not NADH, inhibited the enzyme at concentrations greater than 50 micromolar.

Unlike that of the wild-type, the nitrate reductase from *nar*1a did not bind to blue dextran-Sepharose. The *nar*1a enzyme did bind to Affi Gel Blue, but recoveries were low. The NADH and NADPH nitrate reductase activities of *nar*1a were not separated by affinity chromatography. The nitrate reductase in *nar*1a is a different enzyme than the wild-type NADH nitrate reductase and appears to be a NAD(P)H-bispecific enzyme.

NR[2]-deficient mutants have been isolated from *Arabidopsis thaliana* L. (22), *Nicotiana tabacum* L. (17–20), *Hordeum vulgare* L. (14, 29, 32), and *Pisum sativum* (8, 13). Genetic analysis of *H. vulgare* (14) and biochemical studies of *N. tabacum* (18–20) NR-deficient mutants indicate that NR deficiency is controlled by at least two loci. In barley, these genes appear to be the NR structural gene (*nar1*) and a gene controlling the molybdo cofactor (*nar2*) (14). The genetic control of higher plant NR appears to be similar to that in fungi, particularly *Neurospora crassa* and *Aspergillus nidulans* (5, 9).

Growth rates of NR-deficient mutants of *Arabidopsis thaliana* and *Nicotiana tabacum* are very low with nitrate as the source of nitrogen (20, 22). However, the NR-deficient barley mutants isolated by Warner *et al.* (32) are capable of substantial growth with nitrate as the sole source of nitrogen (21, 31). The barley mutants have low levels of NR (14, 32), which may be the result of leaky mutants or due to the presence of a second NR. Several higher plant species appear to have more than one NR. The major NR in most species is NADH-specific (1), but several species have NAD(P)H-bispecific NR (3, 4, 7, 12, 23, 27, 28). The NR from the wild-type barley is a NADH-specific enzyme (6).

The objective of this study was to characterize the NR from the NR-deficient mutant, *nar*1a, to determine whether the enzyme is the same or different from the wild-type NR.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare* L.) seedlings were grown for 6 to 7 d in a growth chamber at 16°C under continuous light (300 μE $m^{-2}s^{-1}$). The NR-deficient mutants, *nar*1a (formerly Az12) and *nar*2a (formerly Az34), were induced and selected from the cv. Steptoe, as described by Warner *et al.* (32). Both mutants are homozygous and genetically stable (14).

NR Extraction and Assay. Seedlings were extracted with a buffer (3 ml/g fresh weight leaf tissue) containing 25 mM Tris (pH 8.4), 1.5 mM EDTA, 4.0 mM DDT, and 5 μM FAD. The crude homogenates were centrifuged at 27,000*g* for 15 min, and the supernatant was saved (crude extract).

NAD(P)H NR assays were conducted as described previously (30), except that 0.2 ml of a 1:1 mixture of 0.3 mM phenazine methosulfate and 1.0 M zinc acetate was used to stop the reaction (25). To insure complete oxidation of the NAD(P)H, this mixture was vigorously mixed particularly when the NAD(P)H concentrations in the assay were greater than 0.2 mM.

Assays for reduced methylviologen NR activity were performed in a medium containing 25 mM K-phosphate (pH 7.5), 10 mM potassium nitrate, 0.2 mM methylviologen, and 3.2 mM sodium dithionite. Assays were conducted by adding the enzyme to the assay medium (2 ml final volume), incubating at 30°C for 1 min, initiating the reaction with 0.1 ml of the dithionite solution (14 mg/ml of 25 mM phosphate [pH 8.2]), and incubating at 30°C for 30 min. The assay was stopped by vigorously agitating the assay mixture until the methylviologen was oxidized (about 10 s). To reduce interference from substances derived from sodium dithionite, 0.2 ml of 1.5% (v/v) formaldehyde in H_2O was added and incubated for 5 min before nitrite determinations were made (26).

NR Isolation. A 20 to 60% ammonium sulfate fraction was prepared by adding a 0.25 volume of a solution containing 25 mM Tris (pH 7.5) and 1.5 mM EDTA saturated with ammonium sulfate to the crude extract, centrifuging at 27,000*g* for 20 min, and discarding the precipitate. An equal volume of the saturated ammonium sulfate preparation was then added to the supernatant and centrifuged, as described above. The supernatant was discarded, and the precipitate was resuspended in a volume of buffer

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[2] Abbreviations: NR, nitrate reductase; FAD, flavin adenine dinucleotide; LDH, lactate dehydrogenase.

(25 mM Tris [pH 8.0], 1.5 mM EDTA, 7.5 μM FAD, and 1.7 mM DTT) equal to the original volume of crude extract. Approximately 18 ml of this preperation were layered on top of a 5-ml column containing 4 ml of Affi Gel Blue (Bio Rad Laboratories) affinity medium which had been equilibrated previously with the same buffer used to resuspend the ammonium sulfate pellet. After application of the sample, the column was washed with 50 ml of buffer I (25 mM Tris [pH 8.0], 1.5 mM EDTA, 7.5 μM FAD, and 0.5 mM DTT), followed by a 15-ml wash with buffer I containing 0.1 M KCl and a final wash with 15 ml of buffer II (buffer I plus 0.1 M KCl and 0.5 mM $NaNO_3$). The NR was then eluted with 50 ml of a 0- to 250-μM NADPH gradient in buffer II, followed by an elution with 30 ml of buffer II containing 1.5 M KCl. Fractions were collected, and A was determined at 280 nm. Aliquots (0.3 ml) of the first 20 fractions were assayed for NR activity using NADPH as the electron donor, and aliquots (0.3 ml) of the remaining fractions were assayed for methylviologen NR activity. Fractions possessing significant NR activity were saved for further assays using NADH and NADPH. K_m values were determined by Lineweaver-Burk plots in all cases, except for the NADH K_m for NR from Steptoe. In this case, the K_m was so low that accurate results could not be obtained by this method, and the method of Halwachs (10) was used instead.

Table I. *NADH and NADPH NR Activities in Crude Extracts of Two NR-Deficient Mutants and the Steptoe Wild-Type*

NR assays were conducted at pH 7.5 with 0.1 mM NADH, with 0.1 mM NADPH, or with 0.1 mM NADH and 0.1 mM NADPH.

Genotype	Seedling Age	NR Activity		
		NADH	NADPH	NADH + NADPH
	d	*μmol NO_2^-/g fresh wt·h*		
*nar*1a	6	0.4	1.8	1.6
	8	0.3	1.4	1.2
	10	0.2	0.4	0.3
*nar*2a	6	1.2	0.1	1.1
	8	0.6	0.1	0.5
	10	0.5	0.1	0.5
Steptoe	6	19.4	0.2	19.6
	8	10.9	0.1	10.7
	10	11.9	0.3	12.1

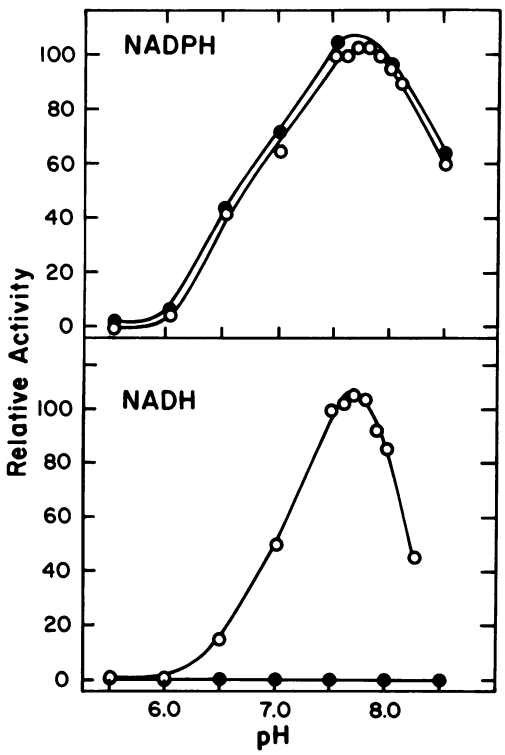

FIG. 1. Influence of pH upon NADH and NADPH NR activities in crude extracts of *nar*1a seedlings. Assays were conducted at the indicated pH in a buffer containing 25 mM Tris, 25 mM K-phosphate, 25 mM maleic acid, and 10 mM KNO_3, with 0.2 mM NADH or 0.1 mM NADPH as the reductant. All assays were conducted in the presence (●) or in the absence (○) of 5 mM pyruvic acid and 50 μg LDH to rapidly oxidize NADH in the assay medium.

RESULTS

The NADPH NR activity relative to NADH NR in crude extracts of Steptoe and the NR-deficient mutant *nar*1a are very different (Table I). In *nar*1a, the NADPH NR activity is 2 to 4 times the NADH activity, while, in Steptoe, the NADPH NR activity is less than 5% of the NADH activity. Furthermore, the NADPH NR activity in *nar*1a is greater than is the NADPH NR activity in Steptoe but is less than 10% of the Steptoe NADH NR activity. Inclusion of both NADH and NADPH in the assay medium did not enhance NR activity and, perhaps, caused a slight inhibition (Table I). In *nar*2a, the NADH NR activities were less than 10% of Steptoe, but, unlike, *nar*1a, *nar*2a had greater NADH than NADPH NR activities. In this respect, *nar*2a resembled Steptoe. *Nar*1 and *nar*2 are not allelic and are presumed to be the NR structural gene and a molybdo cofactor gene, respectively (14). Eight other *nar*1 mutants also had greater NADPH than NADH NR activity (data not presented).

The pH optimum of the *nar*1a NR was approximately 7.7, with NADH or with NADPH as the reductant (Fig. 1). Inclusion of LDH and pyruvate in the assay medium to remove NADH competitively (6) eliminated all NR activity when NADH was used as the reductant but had no significant effect upon NADPH NR activity.

Attempts to purify the *nar*1a NR by affinity chromatography met with only limited success. Unlike the wild-type NR, which has been purified by blue dextran-Sepharose affinity chromatography in our laboratory (15), the *nar*1a NR did not bind well to blue dextran-Sepharose. The *nar*1a NR, however, did bind to Affi Gel Blue and withstood washing with several buffers, including a buffer containing 0.1 M KCl (Fig. 2). A great many of the 280-nm absorbing substances were eluted with these washes without the elution of detectable NR activity (Fig. 2). A NADPH gradient (0–250 μM) resulted in the elution of only a trace amount of NR. Most of the NR recovered was eluted with 1.5 M KCl (Fig. 2); however, the NR recovered from the affinity column was usually less than 10% of the total activity in the crude extract (Table II). The NR recovered from the column retained both the NADH and NADPH NR activities, although the NADPH-to-NADH NR activity ratio appeared to decrease somewhat. The NR recovered from the affinity column was rather unstable and of insufficient quantity to permit kinetic analyses. Attempts to use larger columns which required longer running times resulted in even lower enzyme recoveries. Therefore, the ammonium sulfate-fractionated NR preparation from *nar*1a was used for determination of apparent substrate K_m values.

The NR from *nar*1a had apparent K_m values for nitrate which were approximately 10 times greater than the Steptoe NR K_m for nitrate (Fig. 3). The *nar*1a NR K_m values for nitrate, with NADH and NADPH as the reductant, were 1.5 and 1.2 mM, respectively. The Steptoe NR K_m for nitrate was 0.13 mM. Similar differences in apparent K_m for NADH were observed also. The apparent K_m values for NADH were 110 and 12 μM for the *nar*1a and Steptoe enzymes, respectively (Fig. 4). The *nar*1a NR apparent NADPH K_m (170 μM) was similar to the NADH K_m (110 μM). However, NADPH inhibited the *nar*1a NR at concentrations greater than

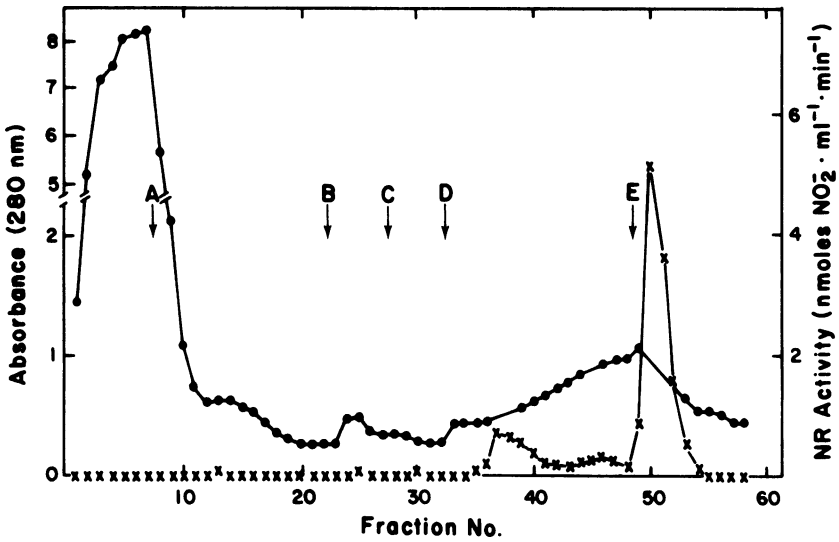

FIG. 2. Affinity chromatography of *nar*1a NR. The column (Affi Gel Blue), crude extract and ammonium sulfate fractionation, and resuspended pellet were prepared as described in "Materials and Methods." Eighteen ml of the resuspended pellet were applied to the column. Arrows indicate where the various wash or elution buffers were applied to the column: A, 50 ml of buffer I (0.025 M Tris [pH 8.0], 1.5 mM EDTA, 7.5 μM FAD, 0.5 mM DTT): B, 15 ml of buffer I to which 0.1 M KCl was added; C, 15 ml of buffer II (buffer I plus 0.1 M KCl and 0.5 mM $NaNO_3$); D, 50 ml of a 0- to 250-μM NADPH linear gradient in buffer II; and E, 30 ml of buffer II, except that KCl was increased to 1.5 M. Forty-drop fractions (about 3.1 ml) were collected at a flow rate of about 0.75 ml/min. A at 280 nm (●) and NR activity (×) were determined as described in "Materials and Methods."

Table II. *Recovery of NAD(P)H NR from Affi Gel Blue Affinity Chromatography*

Results are the average of six experiments. Assays were conducted at pH 7.5 with 0.2 mM NADH or 0.2 mM NADPH.

Fraction	Recovery	NADPH/NADH Activity
	%	*ratio* ± *SD*
100	1.37 ± 0.17	
$(NH_4)_2SO_4$ pellet	110	1.30 ± 0.14
Affi Gel Blue fractions 49–53	10	0.80 ± 0.25

50 μM, while NADH had little or no inhibitory effect at concentrations of at least 400 μM.

DISCUSSION

The NR from the mutant *nar*1a had different properties than did the NR from the wild-type (Steptoe). The pH optima for the NADH and NADPH NR activities of *nar*1a were both approximately 7.7 (Fig. 1), while the pH optima for the wild-type NR were 7.5 and 6 to 6.5 for the NADH and NADPH activities, respectively (6). The substrate apparent K_m values of the *nar*1a NR were all about 10 times larger than were the Steptoe NR K_m values (Figs. 3 and 4). These results indicated that at least a portion of the NR activity from *nar*1a seedlings was due to a different enzyme than that found in the wild-type.

The NR in *nar*1a was capable of using NADPH as an electron donor. At pH 7.5, the activity of the purified wild-type NR with NADPH was less than 1% of the activity with NADH (6), while the activity of the *nar*1a NR was greater with NADPH than it was with NADH (Table I). Crude extracts of wild-type seedlings have enzymes capable of converting NADPH to NADH, which results in artifically high NADPH NR activities. However, most, if not all, of the NADPH NR activity resulting from this conversion was eliminated by inclusion of LDH and pyruvate in the assay medium (6). Presumably, these enzymes are also present in crude extracts of *nar*1a seedlings. However, the NADPH NR activity in *nar*1a crude extracts was not affected by LDH and pyruvate (Fig. 1).

That the *nar*1a NADPH and NADH NR activities were properties of a single NAD(P)H-bispecific NR was indicated by similarities in pH optima (Fig. 1), the lack of increased NR activity when assayed in the presence of both NADH and NADPH (Table I), and the similarities in apparent K_m for nitrate with NADH or NADPH as the reductant (Fig. 3). In addition, the apparent K_m for NADH and NADPH were also similar (Fig. 4). The similarities in *nar*1a NADH and NADPH NR K_m values were particularly significant when compared with the much lower K_m values of the wild-type and other higher plant NADH NR (2). Furthermore, we were not able to separate the NADH and NADPH NR activities by affinity chromatography, although these results should be viewed with caution, because of the difficulties encountered in eluting NR from Affi Gel Blue and the low recovery achieved.

Some of the properties of the *nar*1a NR were similar to those reported for bispecific NR from other species. In higher plants, the NR capable of using NADPH are bispecific and have higher activity with NADPH than with NADH (3, 4, 7, 12, 23, 27, 28). Mutant *nar*1a has higher NR activity with NADPH than it does with NADH (Table I). The bispecific NR from *Erythrina senegalensis* (28) and soybean (3, 12) have rather low affinities for nitrate, with K_m values similar to the *nar*1a NR K_m for nitrate (Fig. 3). The high apparent K_m values for NADH and NADPH, however, were considerably higher than those reported for bispecific NR from soybean (3, 12), *E. senegalensis* (28), and *Dunaliella parva* (11). However, the K_m values for the bispecific NR from *D. tetiolecta* of 100 and 180 μM for NADPH and NADH, respectively (16), are similar to the K_m values for the *nar*1a enzyme (Fig. 4).

Some of the NADH and NADPH characteristics of the *nar*1a NR may indicate that the NADH and NADPH NR activities are due to different enzymes. Some variation in the NADPH to NADH NR activity ratios was observed with seedlings of different ages (Table I) and during different stages of purification (Table II). These variations could have been caused by the presence of two enzymes or, perhaps, by the presence or absence of an unidentified effector. The other concern was the inhibition by NADPH but not by NADH (Fig. 4). The effects of NADPH and NADH could be properties of a single enzyme, if NADPH inhibits or regulates the enzyme by binding at a site remote from the catalytic site, while the catalytic site can accept either NADH or

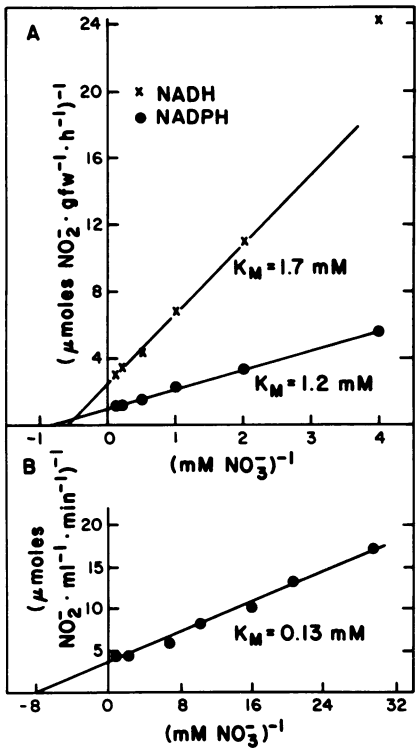

FIG. 3. Activity of *nar*1a and Steptoe NR as a function of nitrate concentration. NR from *nar*1a leaves was extracted, precipitated with ammonium sulfate, and resuspended, as described in "Materials and Methods." Aliquots (0.3 ml) were assayed at the indicated nitrate levels with 0.2 mM NADPH or NADH (A). Steptoe NR, which had been purified by the method of Kuo *et al.* (14) and stored in glycerol, was dialyzed against three changes of 25 volumes of buffer (25 mM Tris [pH 8.0], 1.5 mM EDTA, 7.5 μM FAD, and 1.7 mM DTT) for 15 to 30 min and then diluted 7.5-fold with the dialysis buffer. Aliquots (0.03 ml) were assayed at the indicated nitrate concentration with 0.2 mM NADH (B).

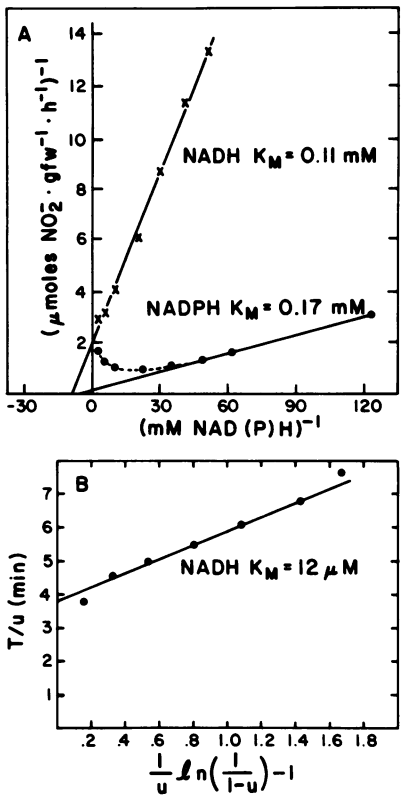

FIG. 4. Activity of *nar*1a and Steptoe NR as a function of NADH or NADPH concentration. The *nar*1a NR was prepared as described in Figure 3. Aliquots (0.3 ml) were assayed at 10 mM nitrate and the indicated NADPH or NADH levels (A). The purified Steptoe NR was prepared as described in Figure 3 and diluted 10-fold with a buffer containing 25 mM Tris (pH 8.0), 1.5 mM EDTA, 7.5 μM FAD, 0.5 mM DTT, and 1 mg/ml BSA. An aliquot (0.03 ml) was assayed in 25 mM phosphate (pH 7.5), 1 mM sodium nitrate, and 7.3 μM NADH, in a final volume of 1 ml, by following the decrease in A_{340} in a Beckman double-beam recording spectrophotometer. A second aliquot of NADH was added to show that enzyme inhibition or denaturation had not occurred. The method of Halwachs (10) was used to compute (T, time in minutes; u, the fraction of NADH used at T) an apparent K_m of 12 μM for the Steptoe NR (B).

NADPH as a substrate. Rigano *et al.* (24) observed that NR from *Cyanidium caldarium* was inhibited by NADH or NADPH concentrations greater than 100 μM and suggested that the enzyme may have a regulatory site distinct from the catalytic site. To our knowledge, cofactor inhibition has not been reported previously for higher plant NR, and any conclusions relating to this characteristic must await further purification and characterization of the enzyme.

Although the *nar*1a NR has some unusual characteristics, the NADH and NADPH NR activities of *nar*1a are probably the properties of a single NAD(P)H-bispecific NR. The possibility that a single mutation in the NR structural gene (*nar*1) could cause all of the differences between the wild-type NR and *nar*1a is very remote, particularly since other *nar*1 alleles also have elevated NADPH NR activities.

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