

Nitrate Reductases from Wild-Type and *nr*₁-Mutant Soybean (*Glycine max* [L.] Merr.) Leaves¹

I. PURIFICATION, KINETICS, AND PHYSICAL PROPERTIES

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ABSTRACT

NADH:nitrate reductase (EC 1.6.6.1) and NAD(P)H:nitrate reductase (EC 1.6.6.2) were purified from wild-type soybean (*Glycine max* [L.] Merr., cv Williams) and *nr*₁-mutant soybean plants. Purification included Blue Sepharose- and hydroxylapatite-column chromatography using acetone powders from fully expanded unifoliolate leaves as the enzyme source.

Two forms of constitutive nitrate reductase were sequentially eluted with NADPH and NADH from Blue Sepharose loaded with extract from wild-type plants grown on urea as sole nitrogen source. The form eluted with NADPH was designated *c*₁NR, and the form eluted with NADH was designated *c*₂NR. Nitrate-grown *nr*₁ mutant soybean plants yielded a NADH:nitrate reductase (designated *i*NR) when Blue Sepharose columns were eluted with NADH; NADPH failed to elute any NR form from Blue Sepharose loaded with this extract. Both *c*₁NR and *c*₂NR had similar pH optima of 6.5, sedimentation behavior (*s*_{20,w} of 5.5-6.0), and electrophoretic mobility. However, *c*₁NR was more active with NADPH than with NADH, while *c*₂NR preferred NADH as electron donor. Apparent Michaelis constants for nitrate were 5 millimolar (*c*₁NR) and 0.19 millimolar (*c*₂NR). The *i*NR from the mutant had a pH optimum of 7.5, *s*_{20,w} of 7.6, and was less mobile on polyacrylamide gels than *c*₁NR and *c*₂NR. The *i*NR preferred NADH over NADPH and had an apparent Michaelis constant of 0.13 millimolar for nitrate.

Thus, wild-type soybean contains two forms of constitutive nitrate reductase, both differing in their physical properties from nitrate reductases common in higher plants. The inducible nitrate reductase form present in soybeans, however, appears to be similar to most substrate-induced nitrate reductases found in higher plants.

in rice (27), corn scutellum (5, 8), barley (7), and soybean (4, 13, 14, 22). The NR activity of soybean, unlike that of other plant species, appears to have NADPH-NR activity as a major component (4, 13). Soybean NADPH-NR and NADH-NR have been separated using DEAE-cellulose (13) and Blue Dextran Sepharose (4). Using these methods, the NADPH-NR was found to have a pH optimum of 6.5, and a Michaelis constant of 5 mM for nitrate. Conejero *et al.* (6) used differential extraction of soybean leaf tissue to separate NR forms and confirmed the Michaelis constants reported by Jolly *et al.* (13). A physiological approach for the separation of NR activities using norflurazon has been chosen by Kakefuda *et al.* (14), who found a NR isoform with a pH optimum of 7.5. Using a NR soybean mutant (*nr*₁) grown on nitrate, and the wild type grown on urea, Nelson *et al.* (22) have been able to characterize constitutive and inducible NR forms in separate plants. Constitutive NR activity has been shown to be associated with the evolution of nitrogenous gasses from leaf discs under anaerobic conditions (12, 21, 26). This gaseous compound has been recently reported to be acetaldehyde oxime (19). The *nr*₁ mutant lacks both constitutive NR activity and gas evolution, but has normal xanthine dehydrogenase activity (21, 22, 26). Genetic analyses showed that the absence of constitutive NR activity is controlled by a single, recessive, nuclear gene (26). The mutation could therefore be either in a structural or a regulatory gene of the apoprotein of the constitutive NR. Constitutive NR activity of the wild-type soybean was reported to have a pH optimum of 6.8 and a Michaelis constant for nitrate of 0.16 mM, while the inducible NR activity from *nr*₁ had a pH optimum of 7.5 and a Michaelis constant for nitrate of 0.14 mM (22). These results are in accordance with those of Kakefuda *et al.* (14), but are in conflict with some biochemical data (13).

An attempt to reconcile these differences using 'western blots' with soybean NR antibodies established that a constitutive NR was identical to NADPH-NR, and that inducible NR was NADH-NR obtained by affinity chromatography from nitrate-grown wild-type plants (25). The difference between the Michaelis constants for nitrate, however, remained unsolved.

The purpose of our investigation was (a) to purify NRs from wild-type soybean (cv Williams) and from *nr*₁ mutants selected from mutagenized Williams soybeans, and (b) to characterize the purified NRs in order to bridge the gap between the biochemical data obtained with Prize soybean (13) and the physiological data obtained with Williams and *nr*₁ soybean plants (22).

MATERIALS AND METHODS

Plant Material. Soybean (*Glycine max* [L.] Merr.) seed from the *nr*₁ mutant was at least four generations removed from the

The NR³ enzyme of most higher plants uses NADH as electron donor to reduce nitrate to nitrite (1, 9). This ubiquitous enzyme has a pH optimum around 7.4, and Michaelis constants for nitrate and NADH of approximately 200 and 2 μM, respectively (1, 9). In higher plants, NADPH-NR activities have been found

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³ Abbreviations: NR, nitrate reductase; FAD, flavin adenine dinucleotide; *c*₁NR and *c*₂NR, constitutive nitrate reductases sequentially eluted from Blue Sepharose with NADPH and NADH, respectively, *i*NR, inducible nitrate reductase.

last generation of mutagenized wild-type (cv Williams) seed. Urea-grown plants were grown hydroponically in a growth chamber as described by Nelson *et al.* (22). Nitrate-grown plants were grown hydroponically as above or in a greenhouse using a gravel hydroponics system similar to the field system described by Harper (11). Fully expanded unifoliolate leaves were used as enzyme source (10- to 13-d-old plants).

Acetone Powder. Freshly harvested leaves were weighed, frozen with liquid N₂, and imbibed with 10 volumes of cold (-18°C) acetone. Leaves were left in acetone in the freezer for 30 to 60 min and then blended in a blender for 30 s, poured into a Büchner funnel lined with filter paper (Whatman No. 1)⁴, and washed with an equal amount of cold acetone. The residue in the funnel was then dried *in vacuo* until it had turned into a fine powder. The powder was stored at -18°C until use.

Enzyme Extraction. Forty-eight g acetone powder, corresponding to approximately 300 g fresh weight, were blended with 3 L extraction medium (100 mM phosphate, 1 mM EDTA, 10 mM cysteine, 7 μM FAD, 0.5% (w/v) casein, final pH 7.4) until in suspension, using a Sorvall Omni-mixer in a 4-L beaker. Excessive foam was repressed by adding 0.1 ml octyl alcohol. The homogenate was first filtered through four layers of cheesecloth, and subsequently through Miracloth. The filtrate was centrifuged for 10 min at 25,000g. The supernatant was used as enzyme source for purification; its activity is referred to as initial activity.

Blue Sepharose Column Chromatography. Blue Sepharose CL-4B was prepared according to Boehme *et al.* (2). Supernatant from acetone-powder extracts were suspended with 75 g, suction-dried Blue Sepharose CL-4B (24). Columns loaded with extract from urea-grown wild-type plants were sequentially eluted with NADPH and NADH (25). NR activity eluted from these columns with NADPH was pooled and designated c₁NR, and that eluted with NADH was designated c₂NR. The c₁NR and c₂NR fractions were purified and stored separately during subsequent procedures. Columns loaded with extract from nitrate-grown nr₁-mutants were eluted as above (25). Active fractions were found in the NADH-eluate only; this NR activity was used for further purification and designated iNR.

Hydroxylapatite Column Chromatography. The c₁NR or c₂NR fractions from Blue Sepharose were pumped directly onto a hydroxylapatite (Calbiochem, San Diego) column (1.4 × 5 cm). The iNR fraction from Blue Sepharose was diluted with ¼ volume of a solution containing 1 mM DTT and 7 μM FAD and then passed through hydroxylapatite as for constitutive NR fractions. Columns were washed with 75 ml buffer of the same ionic strength as the loaded solutions, and then eluted with a linear gradient of 0 to 0.3 M phosphate in equilibration buffer (100 mM phosphate, 1 mM EDTA, 1 mM DTT, 7 μM FAD, final pH 7.4 for c₁NR and c₂NR; 80 mM phosphate, 0.8 mM EDTA, 1 mM DTT, 7 μM FAD, pH 7.4 for iNR). Flow rates were 2.5 ml/min.

Ammonium Sulfate Precipitation. Solid (NH₄)₂SO₄ (300 g/L) was added to pooled active fractions from the hydroxylapatite column and stirred for 30 min. The enzymes were either stored in (NH₄)₂SO₄ solution at -18°C, or collected in a minimal amount of equilibration buffer after centrifugation of the (NH₄)₂SO₄ precipitate for 30 min at 40,000g. The resuspended enzyme preparations were also stored at -18°C.

Sucrose Density Gradients. Linear gradients (5–20%) were prepared following the method of Martin and Ames (17). Sucrose stock solutions were prepared in equilibration medium containing DTT for analysis of NRs, and without DTT for analysis of

Cyt *c* reductase activities. Centrifugation was performed for 19 h at 39,500 rpm in a Beckman L5-75B ultracentrifuge (Beckman Instruments, Palo Alto, CA) using a SW 40 TI swinging bucket rotor (tube size, 1.3 × 9.5 cm). Gradients were fractionated collecting eight drops per tube. Fractions were assayed for enzyme activities and the presence of myoglobin. Svedberg values were calculated according to Martin and Ames (17).

PAGE. Gels (7.5%) and stacking gels (4.5%) were prepared and electrophoresed as described by Smarelli and Campbell (28). NR activity in the gels was detected by incubation of 2-mm slices in regular NR assays containing 0.7 μM FAD. Protein was visualized using Coomassie Brilliant Blue.

Enzyme Assays. NR activity was measured as described by Jolly *et al.* (13), except that FAD and cysteine were omitted from assays. Final nitrate concentrations were 10 mM (NADH-assay) and 80 mM (NADPH-assay) as used by Campbell (4). Michaelis constants for nitrate were calculated from Eadie-Hofstee plots using regular NR assays and by monitoring NAD(P)H oxidation at 340 nm in a final volume of 0.5 ml. Michaelis constants for NAD(P)H were determined according to Halwachs (10). Cyt *c* reductase activity was determined according to Wray and Filner (30) in a final volume of 0.5 ml. Activities were calculated using the extinction coefficients reported by Margoliash and Walasek (16).

Protein. Protein was determined according to Bradford (3).

RESULTS

A NADPH-NR activity was eluted with NADPH from Blue Sepharose when wild-type plant extracts were used (Fig. 1, A and C). This NADPH-NR had a higher activity with NADPH at pH 6.5 than with NADH at either pH 6.5 or 7.5, and regardless of whether the plants were grown on urea or on nitrate (Fig. 1, A and C). No measurable NR was eluted with NADPH from Blue Sepharose loaded with extract from nr₁ mutant plants (Fig. 1B).

A NADH-NR (pH optimum 6.5) was eluted with NADH from Blue Sepharose loaded with extract from wild-type plants grown on urea (Fig. 1C). However, NADH-NR with pH optimum of 7.5, was eluted with NADH when extracts from nitrate-grown mutant plants were the enzyme source (Fig. 1B). The activity eluted with NADH from a column loaded with extract from nitrate-grown wild-type plants had a slightly higher activity at pH 7.5 than at pH 6.5 (Fig. 1A), suggesting that this elution was a mixture of the nitrate-induced enzyme present in the mutant, and the constitutive NADH-enzyme present in wild-type plants. From these elution patterns, it was concluded that there may be both NADPH and NADH-preferring constitutive NR forms present in wild-type Williams soybeans.

Constitutive NR activities showed different affinities to Blue Sepharose. During a 15-min binding, approximately 50% of the NADPH activity, but only 10 to 20% of the NADH activity, was bound to the Blue Sepharose (data not shown). Interconversion of the two constitutive activities did not occur *in vitro*, since rechromatography of NADPH and NADH eluates yielded a single peak for each experiment (data not shown). Due to the presence of two constitutive forms, the purification protocol was redesigned to investigate both constitutive NR activities, designated c₁NR and c₂NR (Table I). The use of Blue Sepharose and hydroxylapatite resulted in a 400- to 800-fold purification of NRs from soybean leaves (Table I and II). The preparations had specific activities of 5.0, 9.5, and 9.4 μmol min⁻¹ mg⁻¹ protein for the c₁NR (NADPH-NR), c₂NR (NADH-NR), and the inducible NR (iNR, NADH-NR), respectively. Overall recoveries ranged from 3% (iNR) to 12% (c₁NR). The relatively good recovery of c₁NR can be attributed to its high affinity for Blue Sepharose, while the low recovery of iNR from the mutant is probably due to poor stability of this enzyme. Initial specific activity was higher in the mutant than in the wild-type (Tables I

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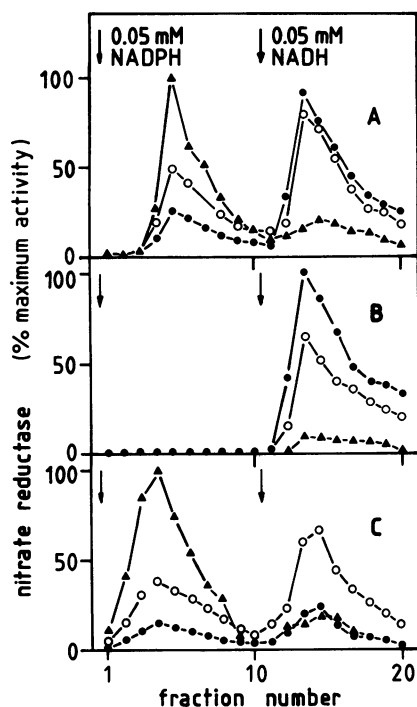


FIG. 1. Elution profiles of nitrate reductase activities from Blue Sepharose. Blue Sepharose (7.5 g) was mixed with extracts, washed with buffer (100 mM phosphate, 1 mM EDTA, 1 mM DTT, 7 μ M FAD, and adjusted to pH 7.4), packed into a column, and eluted with 30 ml 0.05 mM NADPH in buffer, followed by 30 ml 0.05 mM NADH in buffer. Extracts were made with acetone powder obtained from 30 g of leaves. Leaf sources were: A, Williams plants grown on nitrate; B, nr₁ mutants grown on nitrate; C, Williams plants grown on urea. (\blacktriangle — \blacktriangle), NADPH:NR-assay, pH 6.5; (\circ — \circ), NADH:NR-assay, pH 6.5; (\bullet — \bullet), NADH:NR-assay, pH 7.5. Maximum activities were: A, 72 nmol min⁻¹ ml⁻¹; B, 60 nmol min⁻¹ ml⁻¹; C, 66 nmol min⁻¹ ml⁻¹.

and II), likely due to higher light intensities during greenhouse growth of the mutant on nitrate compared with the growth of chamber-grown wild-type plants on urea. The different growth conditions did not seem to alter the proportion of NR, since pH optima, Michaelis constants, and elution patterns from Blue Sepharose were the same for plants grown in the greenhouse or in a growth chamber (data not shown).

The sedimentation behavior of the NRs was studied using sucrose density gradient centrifugation in the range of 5 to 20% sucrose (Fig. 2). Constitutive forms had S values ($S_{20,w}$) of 5.6 (c_2 NR) and 6.0 (c_1 NR). The iNR had a S value of 7.6, cosedimenting with alcohol dehydrogenase. Thus, c_1 NR and c_2 NR

appeared to be smaller than iNR. Absolute values of mol wt could not be calculated from S units, since the postulated asymmetric shape of NR affects its sedimentation behavior. Gradients without sulphhydryl reducing agents were run and assayed for Cyt c reductase activity, and essentially the same profiles were obtained, indicating that this dehydrogenase activity was associated with NR (data not shown). There seemed to be little breakdown of NR, since only one peak of Cyt c reductase activity was found.

PAGE of the purified preparations revealed two major protein bands (Fig. 3). One, migrating 4 cm from the origin, was probably a casein contaminant. This band was in the same position in all the preparations, and it had no nitrate reductase activity (Fig. 3). The other band migrated approximately 2 cm (c_1 NR), 1.8 cm (c_2 NR), and 1 cm (iNR). These bands were associated with NR activity (Fig. 3). The c_1 NR and c_2 NR had similar electrophoretic mobility, and both migrated further than iNR (Fig. 3). Considerable losses in activity occurred during electrophoresis. The loss in activity differed among the three preparations, iNR retaining more activity than the others, while c_2 NR lost almost all its activity (Fig. 3 legend).

While electrophoretic mobility and sedimentation behavior were similar for c_1 NR and c_2 NR, the affinity for nitrate and pyridine nucleotides differed considerably between the two constitutive forms (Table III). The c_1 NR had a high Michaelis constant for nitrate (5 mM), regardless of the electron donor. The c_2 NR had a low Michaelis constant for nitrate (0.19 mM). This latter value was close to the 0.13 mM obtained with iNR. Michaelis constants for NADPH (c_1 NR) and NADH (c_2 NR and iNR) were similar (2–3 μ M).

DISCUSSION

Using Blue Sepharose and an improved elution method (24), two forms of the constitutive NR were separated from wild-type soybean plants (cv Williams) grown on urea. The enzyme eluted with NADPH was designated c_1 NR. Recently, this enzyme has been shown immunologically (25) to be identical to the NADPH-NR described by Jolly *et al.* (13) and Campbell (4). The enzyme eluted with NADH using urea-grown Williams wild-type plants was designated c_2 NR. This activity is prevalent in leaf extracts of urea-grown plants, and has been characterized by Nelson *et al.* (22) using desalted leaf extracts. In contrast to corn NR (20), the two constitutive forms were not interconvertible. Comparing the properties of c_1 NR and c_2 NR, we were able to reconcile the discrepancies between biochemical and physiological data obtained by different workers (4, 13, 14, 22). The c_1 NR was more active with NADPH than with NADH, had a relatively high K_m for nitrate, and differed physically from nitrate-induced NR. These data are consistent with the characteristics of the NADPH-NR described by Jolly *et al.* (13) and Campbell (4). The c_2 NR, on the other hand, was more active with NADH than with

Table I. Summary of the Purification of Constitutive Nitrate Reductases (c_1 NR, c_2 NR) from Leaves of Urea-Grown Wild-Type Soybeans. Assays were done at pH 6.5 with 80 mM KNO₃ for NADPH-dependent activity and 10 mM KNO₃ for NADH-dependent activity.

Fraction	c_1 -NR (NADPH-NR)					c_2 -NR (NADH-NR)				
	Total		Specific activity	Purification	Recovery	Total		Specific activity	Purification	Recovery
	Activity	Protein				Activity	Protein			
	μ mol min ⁻¹	mg	μ mol min ⁻¹ mg ⁻¹ protein	-fold	%	μ mol min ⁻¹	mg	μ mol min ⁻¹ mg ⁻¹ protein	-fold	%
Centrifuged homogenate	24.0	4160.0	0.006	1	100	80.8	4505.0	0.018	1	100
Blue Sepharose pooled peak	4.7	4.8	1.016	169	20	5.8	2.0	2.90	161	7
Hydroxylapatite pooled peak	3.0	0.6	5.016	836	12	3.9	0.4	9.51	528	5

Table II. Summary of the Purification of Substrate-Inducible Nitrate Reductase (iNR) from Leaves of Nitrate-Grown *nr₁* Mutant Soybeans

Assays were done at pH 7.5 with 10 mM KNO₃.

Fraction	Total		Specific Activity $\mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$	Purification -fold	Recovery %
	Activity $\mu\text{mol min}^{-1}$	Protein mg			
Centrifuged homogenate	64.3	2991.0	0.022	1	100
Blue Sepharose pooled peak	4.4	1.8	2.47	112	7
Hydroxylapatite pooled peak	1.9	0.2	9.42	428	3

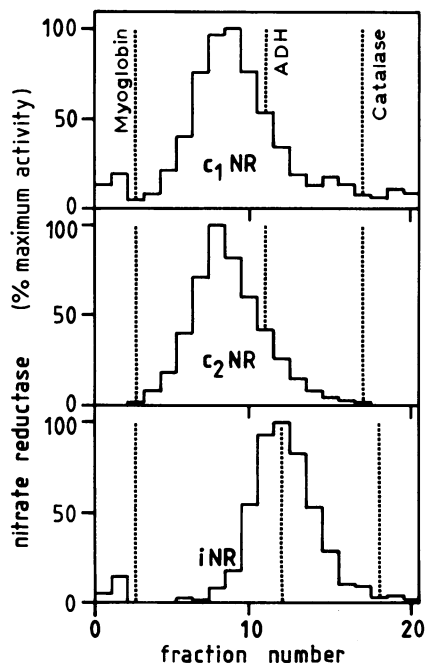


FIG. 2. Sucrose density centrifugation of purified NRs from soybean leaves. Vertical dotted lines represent peaks of marker proteins. ADH, Alcohol dehydrogenase (yeast). Total NR activities loaded onto gradients were: 135 nmol min⁻¹ ml⁻¹ (c₁NR), 125 nmol min⁻¹ ml⁻¹ (c₂NR), and 86 nmol min⁻¹ ml⁻¹ (iNR). Assays were done with NADPH at pH 6.5 (c₁NR), NADH at pH 6.5 (c₂NR), and NADH at pH 7.5 (iNR). Maximum activities were: c₁NR, 8 nmol min⁻¹ ml⁻¹; c₂NR, 15 nmol min⁻¹ ml⁻¹; iNR, 11 nmol min⁻¹ ml⁻¹.

NADPH, had a pH optimum of 6.5, a low K_m for nitrate, and appeared to be approximately the same size as c₁NR. These characteristics are consistent with the reports of Nelson *et al.* (22).

The *nr₁* mutant has been shown to lack constitutive NRs (22), and the mutation has been shown to be in a single, recessive locus (26). Since both c₁NR and c₂NR are absent in the mutant, the expression of both forms may be controlled by the same gene. Immunochemical analyses of mutant extracts (25) have yielded data consistent with the possibility of a mutation in a regulatory gene, but no conclusive statement can be made. Constitutive nitrate reductase activity has been shown to be associated with acetaldehyde-oxime evolution (12, 19, 21). The question remains as to whether c₁NR, c₂NR, both, or even another enzyme regulated by the same gene are responsible for nitrogenous gas evolution.

The *nr₁* mutant has been shown to be a unique tool for studying nitrate-inducible NR in soybeans (22). NADH elution from Blue Sepharose loaded with extract of nitrate-grown Williams plants contained both c₂NR and iNR (Fig. 1A). Therefore,

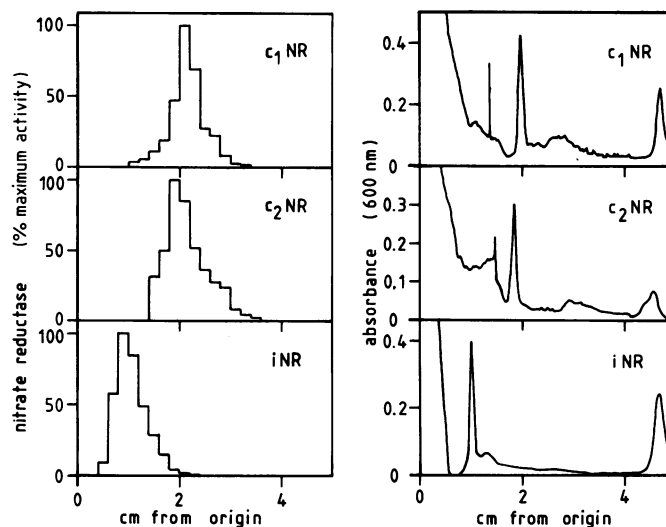


FIG. 3. PAGE of purified NRs from soybean leaves. A 25- μl sample of each purified NR was mixed with 25 μl glycerol and applied to gel tubes (0.5 \times 12 cm). Activities loaded onto gels were: 0.39 $\mu\text{mol min}^{-1} \text{ml}^{-1}$ (c₁NR), 0.88 $\mu\text{mol min}^{-1} \text{ml}^{-1}$ (c₂NR), and 1.54 $\mu\text{mol min}^{-1} \text{ml}^{-1}$ (iNR). Gels were sliced into 2-mm segments and assayed for NR activity. Maximum activities were: c₁NR, 2.3 nmol min⁻¹ ml⁻¹; c₂NR, 0.2 nmol min⁻¹ ml⁻¹; iNR, 5.0 nmol min⁻¹ ml⁻¹. A parallel set of tubes was stained for protein with Coomassie Brilliant Blue R, and scanned at 600 nm wavelength.

Table III. Michaelis Constants (NO₃⁻ and Pyridine Nucleotide) of Purified Nitrate Reductases from Soybean Leaves

	K_m		
	c ₁ NR	c ₂ NR	iNR
NO ₃ ⁻			
With NADPH	4.7 mM	ND ^a	ND
With NADH	5.4 mM	0.19 mM	0.13 mM
NADPH	2.9 μM	26.0 μM	100.0 μM
NADH	11.0 μM	2.9 μM	2.5 μM

^a Not determined.

the mutant was the only viable source of pure nitrate-induced enzyme.

The iNR from soybean was found to have a pH optimum of 7.5 (Fig. 1B), which was similar to the value reported for the purified substrate-induced NR from soybean cotyledons (14). No alteration of iNR seemed to occur during purification, since pH optimum, K_m for nitrate, and preference of NADH as electron donor remained the same as in whole leaf extracts (22). Most higher plant NRs have been shown to be nitrate inducible, and to have optimal activity around pH 7.4 (1). Michaelis constants for nitrate and NADH (Table III), and sedimentation

behavior of iNR (Fig. 2) were also similar to those found for most higher plant NRs (1, 15, 18, 23, 28, 29). Thus, our results confirm earlier claims (22), that iNR from soybeans has similar physical and kinetic properties as most nitrate-inducible NRs from higher plants.

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