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Purification and Characterization of NAD(P)H:Nitrate Reductase and NADH:Nitrate Reductase from Corn Roots[1]

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ABSTRACT

The nitrate reductase activity of 5-day-old whole corn roots was isolated using phosphate buffer. The relatively stable nitrate reductase extract can be separated into three fractions using affinity chromatography on blue-Sepharose. The first fraction, eluted with NADPH, reduces nearly equal amounts of nitrate with either NADPH or NADH. A subsequent elution with NADH yields a nitrate reductase which is more active with NADH as electron donor. Further elution with salt gives a nitrate reductase fraction which is active with both NADH and NADPH, but is more active with NADH. All three nitrate reductase fractions have pH optima of 7.5 and Stokes radii of about 6.0 nanometers. The NADPH-eluted enzyme has a nitrate K_m of 0.3 millimolar in the presence of NADPH, whereas the NADH-eluted enzyme has a nitrate K_m of 0.07 millimolar in the presence of NADH. The NADPH-eluted fraction appears to be similar to the NAD(P)H:nitrate reductase isolated from corn scutellum and the NADH-eluted fraction is similar to the NADH:nitrate reductases isolated from corn leaf and scutellum. The salt-eluted fraction appears to be a mixture of NAD(P)H: and NADH:nitrate reductases.

Nitrate reductase catalyzes the reduction of nitrate to nitrite with pyridine nucleotide in higher plants. Although green tissue generally has higher NR[2] activity than nongreen tissue and is therefore the focus of the majority of NR studies, NR activity has been demonstrated in the roots of many plant species (2, 8, 9, 11, 17, 20). The amount of nitrate reduced in the root, relative to that exported to the shoot, differs greatly among plant species (17). *Xanthium* exports over 95% of the nitrate it takes up to the shoot, whereas in lupin, radish, and pea, the root is the major site of nitrate reduction (17). The ratio of root to shoot nitrate reduction also depends upon the plant's environment, especially the nitrogen nutrition of the plant (2, 9, 15, 17). In addition, the regulation of NR may differ somewhat between species and even between the root and shoot of the same species (9, 15, 17).

NR in roots, like leaf NR, appears to be an inducible enzyme, in that the development of NR activity in root tissue requires the presence of nitrate and the synthesis of RNA and protein (2, 15). The physical properties of root and shoot NR appear to be similar, although not identical (11, 15, 20, 27). Sanderson and Cocking have reported that tomato root NR is similar to tomato leaf NR in its cysteine requirements, its K_m values for nitrate and NADH, and its pyridine nucleotide specificity (20).

In most higher plants, nitrate reduction is catalyzed by NADH:NR (EC 1.6.6.1), an enzyme which is specific for NADH as the electron donor and has a relatively low K_m for nitrate (2). Soybean leaf (3, 10) and cotyledon (16), rice seedling (25) and corn scutellum (4) have an additional NR which is capable of using either NADH or NADPH as the electron donor. This enzyme has been designated NAD(P)H:NR (EC 1.6.6.2) and, in addition to being bispecific, has a higher K_m for nitrate than the NADH:NR. Evans and Nason (8) have found significant NADPH-linked NR activity in the roots of a variety of species. In addition, Elsner (7) has seen evidence for an NADPH-linked NR in corn roots (7). Sanderson and Cocking (20) and Wallace (27), on the other hand, found no evidence for an NADPH-linked NR in tomato and corn roots.

NR from both green and nongreen tissue has been difficult to purify using conventional techniques. The development of an affinity chromatography method using blue-Sepharose has recently allowed for the purification of higher plant NR with both high yield and good specific activity (3–5, 16, 26). In addition to allowing for the purification of higher plant NADH:NR with high yield, affinity chromatography has been useful in separating the two forms of NR present in some tissues (3, 4, 16, 25).

To more fully understand nitrate reduction in higher plants, the properties of NR from all tissues, including roots, must be known. The purification and characterization of root NR has lagged behind the characterization of leaf NR, primarily due to the low levels of NR activity in root extracts and the difficulty encountered in purifying NR. Since the properties of root NR are still largely unknown and a reasonable purification method is now available, we have purified and partially characterized NR from corn roots.

MATERIALS AND METHODS

Plant Material. Corn seeds (*Zea mays* L. cv. W64A×W182E) were soaked in deionized H_2O overnight, then germinated in moist vermiculite in a growth chamber (16 h day/ 25 C). Cool-white fluorescent and incandescent bulbs provided a total irradiance of 27 w/m^2 when measured from 380 to 750 nm with an ISCO SR spectroradiometer. After 4 days, the seedlings were induced with a solution containing 30 mM KNO_3, 20 mM KH_2PO_4, 1 mM $(NH_4)_2SO_4$, 2 mM $Ca(NO_3)_2$, 1 mM $Mg(NO_3)_2$, 0.2 mM Fe-EDTA, and micronutrients. The 5th day, the plants were removed from the vermiculite and the whole roots were washed with tap H_2O, excised, and frozen in liquid N_2.

Extraction and Purification. Fifty to seventy g frozen roots were ground to a coarse powder in a mortar with 0.2 g PVPP/g fresh weight. The powder was then extracted in a blender with 5 volumes of buffer containing 50 mM K-phosphate (pH 7.5), 5 mM cysteine, and 0.5 mM EDTA (14). The resulting slush was filtered through three layers of Miracloth and centrifuged at 17,000*g* for

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[2] Abbreviations: NR, nitrate reductase; PVPP, polyvinylpolypyrrolidone; FAD, flavin adenine dinucleotide; PMS, phenylazine methosulfate; RuBP, ribulose bisphosphate; PEI, poly(ethyleneimine); E_a, energy of activation.

10 min. The crude extract (250–350 ml) was slowly stirred with 10 to 15 ml of blue-Sepharose (5, 16). After 30 min, the blue-Sepharose was collected by vacuum filtration and washed with approximately 100 ml of buffer A containing 100 mM K-phosphate (pH 7.5), 5 mM cysteine, and 0.5 mM EDTA. The blue-Sepharose was suspended in about 50 ml buffer A and packed into a 1.5-cm diameter column. The blue-Sepharose was eluted successively with 50 ml buffer A containing 100 μM NADPH, 10 ml buffer A, 50 ml buffer A containing 100 μM NADH, 10 ml buffer A, and 50 ml buffer A containing 0.4 M KNO_3. Five-ml fractions were collected and, unless otherwise indicated, fractions eluted with NADPH and NADH contained 0.2 ml 0.3 M KNO_3 (final concentration: 13 mM). The two or three fractions having the highest NR activity from each elution were pooled and used for further study. Where corn leaf NR was used, the leaves from 8-day-old corn seedlings were treated in the same manner as the roots. Squash cotyledon and spinach NR were extracted and purified according to the method of Campbell and Smarrelli (5). The purifications were carried out at 4 C and the purified NR were stored on ice until further use.

NR Assays. The root crude extract and blue-Sepharose wash were assayed in a 2-ml volume containing 72 mM Hepes, 8.5 mM K-phosphate (pH 7.5), 11 mM KNO_3, and either 500 μM NADPH or NADH or a mixture of 250 μM NADPH and 250 μM NADH at 30 C. From 200 to 400 μg protein were used in the assay. Nitrite was determined by the method of Scholl *et al.* (22). The purified NR and all leaf NR were assayed in a 2-ml volume containing 72 mM Hepes, 8.5 mM K-phosphate (pH 7.5), 11 mM KNO_3, 10 μM FAD, and either 100 μM NADPH or NADH or a mixture of 50 μM NADPH and 50 μM NADH. About 10 μg protein were used to assay the specifically eluted fractions and about 40 μg protein were present in the assay of the salt-eluted fractions. As the salt-eluted NR contained 0.4 M KNO_3, the final NO_3^- concentration here was 31 mM. Nitrite was determined as above, omitting PMS except where indicated. All the assays were run as 12-min time courses, and included zero time blank to verify the linearity of nitrite production. One unit of NR is defined as 1 nmol NO_2^- produced/min.

NR Decay. The peak fractions from each elution were pooled and brought to the temperature indicated. At various times aliquots of the pools were assayed for NR activity as described above. Decay rates were determined using least squares fit of the log NR activity *versus* time and the half-lives were determined from the slope of the log NR activity *versus* time (26). The activation energies were estimated according to Sherrard and Dalling (26).

pH Optima. The pH optima of the three NR fractions obtained from blue-Sepharose were determined by assaying the peak fractions in a 2-ml volume containing: 25 mM each Mops, Mes, phosphate, and Tris adjusted to the desired pH, 12.5 mM KNO_3, 10 μM FAD, and 100 μM pyridine nucleotide, and enzyme. Nitrite was determined as described earlier.

Apparent K_m Values for Nitrate. The apparent K_m values for nitrate were determined on the NADPH- and NADH-eluted NR, which had been eluted in the absence of nitrate. The NR was assayed as described for the purified NR, except the concentration of KNO_3 was varied from 0 to 20 mM. Excess pyridine nucleotide was oxidized with PMS (22). The apparent K_m values for nitrate were determined using the direct linear plot (5).

Stokes Radius Determination. The Stokes radii of the NR from various sources were determined using an agarose column (Bio-Gel A-1.5m) of 58 × 0.9 cm. A 5-mm layer of Sephadex G-25 was placed on top of the Bio-Gel to aid in sample application. Blue dextran was used to determine the void volume and BSA (Stokes radius = 3.55 nm), aldolase (4.8 nm), catalase (5.2 nm), and RuBP carboxylase (7.3 nm) were used to calibrate the column. One ml of unknown or standard was applied to the column and eluted in buffer B containing 100 mM K-phosphate (pH 7.5), 100 mM KCl, and 0.5 mM EDTA using a descending flow rate of approximately 4 ml/h. Protein was monitored using both a Pharmacia UV-1 monitor and recorder and the A_{280} of the fractions (0.55 ml). NR activity was measured in the fractions as described earlier for partially purified NR. When corn root NR were evaluated, the fractions were assayed using NADH or NADPH as the electron donor. The NR activity peak did not usually coincide with the major protein peak(s) eluted from the column. Standard curves were determined using a least squares fit of $-(\log K_{av})^{1/2}$ *versus* Stokes radius (24) or K_{av} *versus* log mol wt (1). In all cases, the correlation coefficients of the lines were greater than 0.98.

The RuBP carboxylase was extracted from 10-day-old squash cotyledons in 25 mM Bicine (pH 7.9), and 10 mM mercaptoethanol, and concentrated by ammonium sulfate precipitation. The concentrated enzyme was purified by gel filtration on Sepharose 4B-CL, which had been equilibrated with the extraction buffer. One ml of the purified RuBP carboxylase was applied to the Bio-Gel column and eluted in the same manner as the other calibration standards. RuBP carboxylase activity was assayed using the method of Chen *et al.* (6). RuBP carboxylase activity from the Bio-Gel column was found to coincide with the major protein peak.

NADPH Phosphatase Stoichiometry. Aliquots from the pooled NR elutions (no nitrate present in fractions) were incubated in the purified NR assay mixture with and without nitrate for 1.5 to 2 h. Following incubation NAD^+, $NADP^+$, NADH, and NADPH were separated using poly(ethyleneimine)-cellulose TLC (18). Ten μl of the assay mixture were spotted on PEI-cellulose plates which had been previously washed with deionized H_2O and the plates were developed in 1 M LiCl. After development and drying, NADH and NADPH were visualized using a long wave UV lamp. Because NAD^+ and $NADP^+$ do not fluoresce at these wavelengths, and are difficult to detect, the oxidized nucleotides were made fluorescent by suspending the TLC plates above a 1:1 mixture of methyl ethyl ketone and concentrated NH_4OH (21). To quantitate the $NADP^+$ produced, the spots corresponding to $NADP^+$ were cut from the plate and eluted with 1 ml H_2O (21). After approximately 0.5 h, 0.2 ml methyl ethyl ketone and 0.8 ml 2.6 M NH_4OH were added. Five min later, 3 ml 0.85 M HCl was added, and 5 min afterward, the mixtures were incubated in a boiling water bath for 5 min. The mixtures were cooled under running water and the $NADP^+$ concentration was determined with a Fluorometer-A4 (Farrand Optical Co.). The fluorometer was standardized using $NADP^+$ which had been treated in the same manner as the TLC plate eluant.

Protein. Protein was determined according to the method of Lowry *et al.* (12), or by the method of Sedmak and Grossberg (23) using BSA (Sigma fraction V) as a standard.

Sources of Materials. The corn seed was obtained through the Wisconsin College of Agriculture and Life Sciences. The Cibacron blue, used in making blue-Sepharose, was from Polysciences and the Sepharose 4B-CL was from Sigma, as was the BSA. The Bio-Gel A-1.5m was purchased from Bio-Rad Laboratories and the catalase and aldolase used in gel filtration were from Pharmacia. The poly(ethyleneimine)-cellulose was obtained through Brinkman Instruments, Inc. All other chemicals and biochemicals were of reagent grade or better.

RESULTS

Extraction and Assay of Root NR. NR activity in corn root extracts prepared as described was 20 to 40 units/g fresh weight. Since the activity was low and there was a significant amount of nitrite in the crude extract (about 40 nmol/ml extract), zero time controls were always included in the enzyme assay. In the crude extract of roots, high concentrations (500 μM) of pyridine nucleotide were reported to be necessary to obtain maximum NR activity

(14). We also found this to be necessary, although the dependence on high nucleotide concentration did not appear to be a property of the NR, *per se*, but of the extract. When the change in A_{340} was measured in the absence of nitrate, there was a high rate of nucleotide oxidation with root crude extracts and a small, but still significant, oxidation rate with the purified NR fractions. The purified NR, however, required much lower (100 μM) levels of pyridine nucleotide for maximum activity and the high NAD(P)H requirement was not observed with any of the leaf extracts used.

The root crude extracts were capable of using NADPH to support nitrate reduction. This rate was approximately 70% of the nitrate reduction rate with NADH (Table I). The NR activity in the crude extract, in the presence of both NADPH and NADH, was greater than with either nucleotide singly. This indicated the presence of two NR in the crude extract (16). In contrast, NADPH-linked NR activity in corn leaf crude extracts was less than 10% of the NADH:NR activity (Table I). Also, NR activity in leaf extracts was the same in the presence of NADH alone or in a mixture of the two nucleotides. From the results of the NR assays with both nucleotides present, it appeared that there was one NR in corn leaves, as had been reported by others (27), and that there was more than one NR in corn roots.

Purification. Corn root NR, extracted in 50 mM phosphate buffer, bound to blue-Sepharose at 50 to 80 units/ml blue-Sepharose. This binding capacity was somewhat lower than the binding capacity of leaf NR for blue-Sepharose, but this could reflect the low NR concentration in the extract rather than a low affinity of root NR for blue-Sepharose. The NR activities found in the crude extract, the blue-Sepharose wash, and the purified fractions were at least 50% inhibited by 10 μM Cibacron blue (5). After the NR was bound to blue-Sepharose, it was not washed off by buffer, indicating that the NR was tightly bound to the blue-Sepharose. However, not all of the NR in the crude extract bound to the blue-Sepharose. The amount of NR which did not bind was variable and depended on the NR activity of the crude extract, the volume of the crude extract and the amount of blue-Sepharose used.

After the root NR activities were bound to blue-Sepharose and the gel was thoroughly washed, NADPH eluted an NR from the gel which was about equally active with NADH or NADPH as electron donor (Fig. 1 and Tables I and II). This NADPH-eluted NR had a ratio of NADH-linked to NADPH-linked activity of 0.8 ± 0.1, indicating only a slight degree of variability in this fraction. The NR activity of this fraction with both pyridine nucleotides present was about equal to the activity with either nucleotide alone. This indicated a single form of NR could be present if it had about equal affinity for NADH and NADPH.

Table I. *Nitrate Reductase Activities of Corn Tissues*

NR was extracted and purified from corn root or leaf tissue as described. The enzyme was assayed in the presence of NADPH, NADH, or both pyridine nucleotides as indicated.

NR Source	Specific Activity			NADH:NR Activity/ NADPH: NR Activity
	NADH	NADPH	Both	
	units/mg protein			
Root				
Crude extract	8.5	6.0	12	1.4
NADPH-eluted	36	42	44	0.85
NADH-eluted	142	20	148	7.1
Salt-eluted	20	10	22	2.0
Leaf				
Crude extract	11	0.8	10	14
NADPH-eluted	52	16	56	3.2
NADH-eluted	780	27	850	29
Salt-eluted	120	2.5	130	48

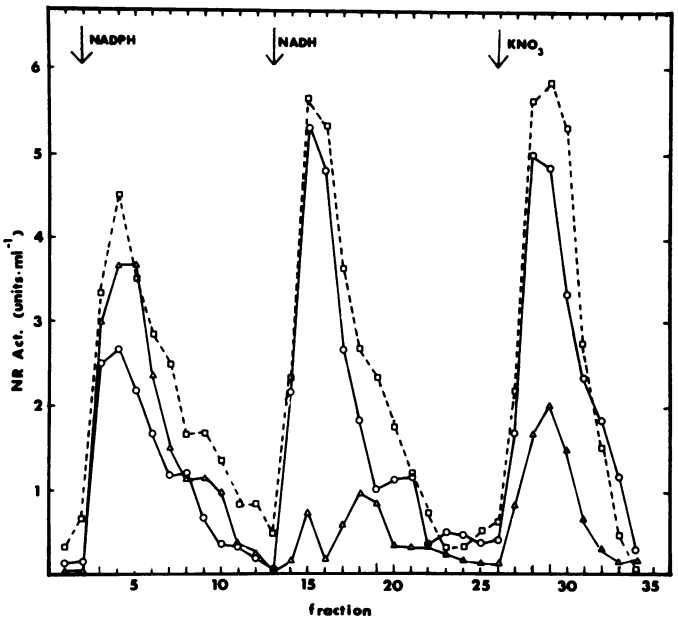

FIG. 1. Elution of corn root NR from blue-Sepharose. Five-day-old corn roots were extracted and treated as described. The NR activity was assayed in the presence of NADPH, △; NADH, ○; or both NADPH and NADH, □.

Subsequent elution of the blue-Sepharose with NADH yielded an NR which was more active with NADH as electron donor and which also showed only a slight additive effect when assayed with NADH plus NADPH. This indicated the presence of a single monospecific form of NR. There was a low and variable NADPH-linked NR activity in this fraction. After elution of the blue-Sepharose with salt, an NR was obtained which used either nucleotide as electron donor. The ratio of NADH- to NADPH-linked NR activity in this fraction was about 2:1, but the ratio varied from one preparation to another. Assaying the salt-eluted NR in the presence of both nucleotides gave activities equal to or greater than the NADH-linked NR activity. This pattern of NR activities could result from either a mixture of two NR or a single bispecific NR with a much lower K_m for NADH than NADPH. This salt elution appeared to release residual NAD(P)H: and NADH:NR from the column, as has been reported previously (4, 16).

Corn leaf NR, when eluted from blue-Sepharose in the same manner as corn root NR, displayed different characteristics (Table I). The NR from all three elutions was more active with NADH as electron donor and, in all cases, the increase in NR activity in the presence of both nucleotides could be accounted for by decreased NADH interference with color development.

While additions of 10 μM FAD had no effect on NR activity in root crude extracts, NR activity in the purified enzymes was stimulated 10 to 20% by assaying with FAD. The NADPH-linked NR activity of the NADH-eluted NR was not stimulated by FAD. In order to maintain constant assay conditions, FAD was used in the assay of all purified NR.

NR Decay. In the crude extract, the NADPH-linked NR activity was less stable than the NADH-linked activity, while the activity with both nucleotides was of intermediate stability (Table III). This difference in stability could be due to a single bispecific NR with differential decay rates for its two activities, which was unlikely in light of the purification data and the NR activity in the presence of both nucleotides. On the other hand, the different stabilities could be due to two NR with different nucleotide specificities and decay rates. The NADPH-eluted NR activities are indeed less stable than the NR activities of the other two

Table II. *Summary of Blue-Sepharose Purification of Corn Root NR*

NR was extracted and purified as described. The enzyme was assayed in the presence of NADH or NADPH as indicated.

	NADH	NADPH	Protein	Specific Activity		Recovery		Purification	
				NADH	NADPH	NADH	NADPH	NADH	NADPH
	total units		*mg*	*units/mg protein*		*%*		*-fold*	
Crude extract	1440	1270	144	10	8.8	100	100	1	1
NADPH-eluted	37	38	0.72	51	53	6.1	7.6	5.1	6.0
NADH-eluted	37	13	0.50	74	26	6.1	2.6	7.4	3.0
Salt-eluted	97	17	4.0	24	4.2	16	3.4	2.4	0.5

Table III. *Decay of NR Activity*

The half-lives and E_a values for decay of the crude extract and purified NR were determined as described.

NR Source	Protein	Electron Donor	Specific Activity	$t_{1/2}$ 0 C	$t_{1/2}$ 25 C	E_a
	mg/ml		*units/mg protein*	*h*		*kcal/mol*
Crude extract	0.5	NADH	11	18.8		
		NADPH	8.8	7.5		
		Both	13	14.7		
NADPH-eluted	0.10	NADH	30	7.7	3.3	5.9
		NADPH	35	7.1	3.3	6.1
		Both	40	5.3	2.5	4.8
NADH-eluted	0.07	NADH	115	27	7.0	8.8
		NADPH	14	7.0	6.0	0
		Both	125	24	8.2	7.1
Salt-eluted	0.4	NADH	25	41	5.8	12.8
		NADPH	10	35	4.3	13.5
		Both	30	99	5.8	18.6

fractions, especially at 0 C. Also, the NADH- and NADPH-linked activities of the NADPH-eluted NR had similar half-lives at both 0 and 25 C. This seems to indicate that a single enzyme species was present, with the same active site being affected with time. The NADH-eluted NR, on the other hand, had a stable NADH-linked activity, while the NADPH-linked activity had a half-life about equal to that of the NADPH-eluted NR and an unusually low E_a for decay. This could mean that the NADPH-linked activity was nonenzymic or that the activity was unrelated to the NADH-linked activity of this fraction. The salt-eluted NR activities were very stable at 0 C, but had similar half-lives to the other fractions at 25 C. The stability at 0 C may have been due to the high concentration of salt (0.5 M) or protein (about 500 μg/ml) in this fraction, relative to the other two.

pH Optima. The pH optima for all of the NR activities were 7.5, like the pH optima for corn scutellum and corn leaf NR (4, 5). Since the pH optimum was 7.5 for both NADH- and NADPH-linked NR activity in the NADPH-eluted NR fraction, the possibility that the NADPH-linked NR activity was due to a contaminating acid phosphatase seemed unlikely. In systems where the NADPH-linked NR activity was due to phosphatase, it was found that the pH optimum for this activity was about 6.2 (7, 27). The NADPH-linked NR activity of the NADH- and salt-eluted NR often had a very broad pH optimum. The NADPH-linked NR activity of these fractions was low and often displayed a nearly constant level of activity over a pH range of 6.5 to 8.0. When the NADPH-linked NR activity of the salt-eluted NR was high, it did display a pH optimum of 7.5.

Apparent K_m Values for Nitrate. The determination of the K_m values for nitrate of the NADPH and NADH-eluted NR were carried out with NR which was eluted in the absence of nitrate. Also, PMS was used to oxidize excess pyridine nucleotide, which could interfere with nitrite determinations, especially at low NR activity (22). The apparent K_m for nitrate of the NADPH-eluted NR was 0.3 mM in the presence of 0.1 mM NADPH and 0.2 mM in the presence of 0.1 mM NADH. The V_{max} of these activities were the same. The NADH-eluted NR had a lower K_m for nitrate of about 0.07 mM in the presence of 0.1 mM NADH, similar to that reported for corn leaf (5). When the nitrate K_m for the NADH-eluted NR was determined in the presence of 0.1 mM NADPH, it was found to be similar to the K_m in the presence of NADH. The low level of activity made estimation of the K_m difficult.

Stokes Radius Determinations. The Stokes radii of the three corn root NR eluted from blue-Sepharose and NADH:NR from corn, squash, and spinach leaves were estimated using gel filtration. The Stokes radii of NR from these sources range from 5.8 to 6.4 nm (Table IV), when determined according to the method of Siegel and Monty (24). It appears from Table IV that estimations of mol wt were highly dependent upon the method of calculation. The corn root NR showed no separation of NADPH- and NADH-linked NR activity after gel filtration.

Phosphatase Assay and Stoichiometry. Because an NADPH-phosphatase could interfere with the assay of NADPH-linked NR activity through the production of NADH (7, 27), the products of nitrate reduction with NADPH were separated and identified using PEI-cellulose TLC. After development of the TLC plates in 1 M LiCl, NADPH, $NADP^+$, NADH, and NAD^+ were separated with R_F values of approximately 0.3, 0.5, 0.58, and 0.75, respectively. The R_F values of $NADP^+$ and NADH did not differ greatly; however, NADH was fluorescent and $NADP^+$ was not, so that NADH production could be detected by irradiating with UV light before derivatizing the $NADP^+$. No NADH or NAD^+ was detected in any of the NADPH-linked NR assays, eliminating the

Table IV. *Stokes Radii and Molecular Weights of NR*

NR Source	Stokes radius[a]	Mol wt[b]	Mol wt[c]
	nm	*daltons* $\times 10^{-3}$	
Corn root			
NADPH eluted	5.8	302	190
NADH eluted	5.8	302	190
Salt eluted	6.0	340	194
Corn leaf	6.0	340	194
Squash cotyledon	6.4	410	207
Spinach	6.0	340	194

[a] Determined by the method of Siegel and Monty (24).

[b] Molecular weight determined by the method of Andrews (1).

[c] Molecular weight determined by the method of Siegel and Monty using the Stokes radius indicated, a sedimentation coefficient of 8.15 (13) and a partial specific volume of 0.725 cm^3/g.

possibility of phosphatase interference.

The $NADP^+$ formed in the NADPH:NR reaction was quantitated by eluting it from the PEI-cellulose plate and forming a fluorescent derivative (21). It was necessary to correct for NADPH oxidation in the absence of nitrate to do this quantitation, as there was significant $NADP^+$ produced in the absence of nitrate. Although the NADPH utilized for nitrate reduction was small compared to the amount oxidized in the control reaction mixture, a good correlation was found between the NADPH oxidized due to nitrate reduction and the amount of nitrite formed. For the NADPH-eluted NR there were 14 nmol $NADP^+$ formed for 14.5 nmol nitrite formed, and in the NADH-eluted NR, there were 6 nmol of $NADP^+$ formed for 4.3 nmol nitrite formed. Thus, the stoichiometry was approximately 1:1 between NADPH oxidized and nitrate reduced.

DISCUSSION

We have isolated and purified the NR of corn roots using the techniques which have been applied to other higher plant NR (4, 5, 16, 25, 26). The root NR will bind to and can be eluted from the affinity media, blue-Sepharose. Unlike the corn leaf NR, two distinctly different NR are obtained from blue-Sepharose by eluting first with NADPH, then with NADH. This, together with the enhancement of NR activity in the crude extract when assayed in the presence of both nucleotides, leads to the conclusion that there are two NR in corn roots. The fact that the enhancement of NR activity is not additive indicates that at least one of the NR in corn roots is bispecific (16). The NR differ in their pyridine nucleotide specificity, their K_m values for nitrate and their relative stability. They have similar stimulation of activity by FAD, pH optima, and Stokes radii.

The NADPH-eluted NR is bispecific, *i.e.* it can use either NADPH or NADH as electron donor. In this way, it is similar to the NAD(P)H:NR of soybean leaf (3, 10) and cotyledon (16), corn scutellum (4), and rice seedlings (25). Therefore, this enzyme is designated corn root NAD(P)H:NR. This relatively unstable enzyme has a pH optimum of 7.5, a Stokes radius of 5.8 nm and an apparent K_m for nitrate of 0.3 mM in the presence of NADPH and 0.2 mM in the presence of NADH. The activity of this NR is stimulated by the presence of FAD.

The NADH-eluted NR is monospecific for NADH as the electron donor. This property is similar to the nucleotide specificity of the NADH:NR of the leaf and scutellum of corn (4, 5), rice seedlings (25), and higher plants in general (2). This enzyme is therefore designated corn root NADH:NR. The NADH:NR appears to have been separated effectively from the NAD(P)H:NR which was not eluted with NADPH. However, there is low and variable NADPH-linked NR activity in this fraction and it is difficult to determine whether this activity is due to a slight contamination by NAD(P)H:NR or to promiscuity of the NADH:NR. The corn root NADH:NR has the same pH optimum and Stokes radius as the NAD(P)H:NR and is stimulated by FAD in the same way as the NAD(P)H:NR. The root NADH:NR has a lower apparent K_m for nitrate than the NAD(P)H:NR. The nitrate K_m of 0.07 mM for the root NADH:NR is the same as the K_m found for corn leaf NR and is similar to other NADH:NR (5). The root NADH:NR also appears to be more stable than the NAD(P)H:NR.

The salt-eluted NR activity seems to be a mixture of the NADH:NR and NAD(P)H:NR. NR from certain tissues, such as corn leaf (5) and wheat leaf (26), binds to blue-Sepharose both specifically and nonspecifically. The specifically bound NR can be eluted with pyridine nucleotide and is presumably bound to the Cibacron blue at the pyridine nucleotide site (5). It appears that both the NAD(P)H:NR and NADH:NR of corn roots bind to blue-Sepharose both specifically and nonspecifically. Thus, elution of the blue-Sepharose column with salt yields the nonspecifically bound NR activities and the characteristics of the NR activity vary depending on the degree to which the two NR are nonspecifically bound.

Both NR from corn roots are similar in molecular size to NR from other sources. The Stokes radii of all of the NR tested is about 6.0 nm. Others (13) have also found the Stokes radius of higher plant NR to be 6.0 nm. Deriving the mol wt of NR from gel filtration data is difficult. The method of Andrews (1) (see Table IV) gives mol wt of 300,000 to 400,000. This is similar to the mol wt of spinach NR suggested by Relimpio *et al.* (19). If the sedimentation coefficient of NR is assumed to be 8.1 *s* (13) and the partial specific volume of NR is assumed to be 0.725 cm^3/g, the mol wt of NR may be calculated by the method of Siegel and Monty (24). This method gives mol wt for NR of about 200,000, or about one-half that derived by the method of Andrews (1). This discrepancy is due to the fact that NR is apparently an asymmetric protein, and therefore, it behaves anomolously on gel filtration (13). The gel filtration properties of NR are inadequate to determine mol wt, but provide one component, the Stokes radius, required to calculate the mol wt (24).

There is evidence for two NR in soybean leaves (3, 10), soybean cotyledons (16), rice seedlings (25), corn scutella (4), and corn roots (Table V). The nitrate-reducing systems from corn tissues and rice seedlings have some common properties, while the systems from soybean tissues are somewhat different. In all of the known systems with two NR's, the NAD(P)H:NR is bispecific, while the NADH:NR is monospecific for NADH. The soybean NR have pH optima of 6.5, while the NR from the other systems have maximum NR activity at about pH 7.5. It is difficult to compare the K_m values of enzymes which have been treated in different ways; the NAD(P)H:NR from corn and from rice seedlings have quite different apparent affinities for nitrate than the soybean leaf NAD(P)H:NR. The soybean leaf NAD(P)H:NR has a remarkably high K_m for nitrate, especially when compared to the NADH:NR from the same tissue. The corn and rice NAD(P)H:NR have lower apparent K_m values and their apparent affinities are only slightly less (3–4 times) than the apparent affinity of their NADH:NR for nitrate (4, 25). The NADH:NR from all of these systems appear to be similar to NADH:NR from other systems with nitrate K_m of 0.1 mM or less (2, 5, 13). We have found that the NADH:NR from the roots and leaves of corn have the same apparent K_m for nitrate. Sanderson and Cocking (20) have reported that the NADH:NR from tomato leaves and roots are similar, although the nitrate K_m values here are somewhat higher than usual (400 μM for the leaf NR and 230 μM for the root NR). The soybean leaf NR show a small difference in molecular size (NAD(P)H:NR = 5.2 nm and NADH:NR = 5.8 nm, recalculated from 10), while the two enzymes from corn root are very similar in size. Thus, it appears that the NAD(P)H:NR from corn scutella

Table V. *Summary of NAD(P)H:NR and NADH:NR Properties*

	Corn Root		Corn Scutellum[a]		Soybean Leaf[b]		Corn Leaf[c]
	NAD(P)H:NR	NADH:NR	NAD(P)H:NR	NADH:NR	NAD(P)H:NR	NADH:NR	NADH:NR
NADH:NRA/NAD(P)H:NRA	0.86	7.1	0.60	5.0	0.85	5.8	50
pH optimum	7.5	7.5	7.5	7.5	6.5	6.5	7.5
Stokes radius, nm	5.8	5.8			5.2	5.8	6.0
K_m, apparent, for NO_3^-, mM	0.3[d]	0.07[e]	0.6[d]	0.2[e]	7.5[f]	0.11[e]	0.07[e]

[a] From Campbell (4).
[b] From Campbell (3) and Orihuel-Iranzo and Campbell (unpublished data).
[c] Campbell and Smarrelli (5).
[d] Apparent K_m was measured in the presence of 0.1 mM NADPH.
[e] Apparent K_m was measured in the presence of 0.1 mM NADH.
[f] True K_m was determined in the presence of NADPH.

and root and from rice seedlings are similar, while the NAD(P)H: NR from soybean has quite different properties.

Nitrate reductase has been demonstrated in the roots of numerous plant species (2, 8, 27), but, the extent to which NAD(P)H: NR contributes to root nitrate reduction requires further investigation. The levels of NR found in extracts of root tissue are often low and some tissues may require special extraction techniques (2) for any NR activity to be demonstrated. For the most part, root NR have been found to have or have been assumed to have the same properties as leaf NR. NADPH-linked NR activity has not been found in extracts of tomato (20) or pea roots (17). Wallace has looked for NADPH-linked NR activity in corn root crude extracts and has found it to be significant only at low pH and in the absence of phosphate (27). Elsner (7), on the other hand, has observed preliminary evidence for NADPH-linked NR activity in corn roots under normal pH and phosphate conditions. It is difficult to reconcile the differing results presented here and those of Wallace. The presence of NAD(P)H:NR in corn roots may be dependent upon cultivar and age, as in soybean cotyledons (16), and/or upon growth and induction conditions, as in rice seedlings (25). Additionally, NAD(P)H:NR may be difficult to detect due to its relative instability. In the system we have used, it appears that, unlike soybean, the NAD(P)H:NR is limited to the nongreen tissues, the root and scutellum, while the NADH: NR is present in all tissues.

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