Plant Physiol. (1988) 88, 0389–0395
0032-0889/88/88/0389/07/$01.00/0

The Conversion of Nitrite to Nitrogen Oxide(s) by the Constitutive NAD(P)H-Nitrate Reductase Enzyme from Soybean[1]

Received for publication January 26, 1988 and in revised form April 14, 1988

JOHN V. DEAN[2] AND JAMES E. HARPER*
Department of Agronomy (J.V.D.) *and United States Department of Agriculture, Agricultural Research Service* (J.E.H.), *University of Illinois, Urbana, Illinois 61801*

ABSTRACT

A two-step purification protocol was used in an attempt to separate the constitutive NAD(P)H-nitrate reductase [NAD(P)H-NR, pH 6.5; EC 1.6.6.2] activity from the nitric oxide and nitrogen dioxide ($NO_{(x)}$) evolution activity extracted from soybean (*Glycine max* [L.] Merr.) leaflets. Both of these activities were eluted with NADPH from Blue Sepharose columns loaded with extracts from either wild-type or LNR-5 and LNR-6 (lack constitutive NADH-NR [pH 6.5]) mutant soybean plants regardless of nutrient growth conditions. Fast protein liquid chromatography-anion exchange (Mono Q column) chromatography following Blue Sepharose affinity chromatography was also unable to separate the two activities. These data provide strong evidence that the constitutive NAD(P)H-NR (pH 6.5) in soybean is the enzyme responsible for $NO_{(x)}$ formation. The Blue Sepharose-purified soybean enzyme has a pH optimum of 6.75, an apparent K_m for nitrite of 0.49 millimolar, and an apparent K_m for NADPH and NADH of 7.2 and 7.4 micromolar, respectively, for the $NO_{(x)}$ evolution activity. In addition to NAD(P)H, reduced flavin mononucleotide ($FMNH_2$) and reduced methyl viologen (MV) can serve as electron donors for $NO_{(x)}$ evolution activity. The NADPH-, $FMNH_2$-, and reduced MV-$NO_{(x)}$ evolution activities were all inhibited by cyanide. The NADPH activity was also inhibited by *p*-hydroxymercuribenzoate, whereas, the $FMNH_2$ and MV activities were relatively insensitive to inhibition. These data indicate that the terminal molybdenum-containing portion of the enzyme is involved in the reduction of nitrite to $NO_{(x)}$. NADPH eluted both NR and $NO_{(x)}$ evolution activities from Blue Sepharose columns loaded with extracts of either nitrate- or zero N-grown winged bean (*Psophocarpus tetragonolobus* [L.]), whereas NADH did not elute either type of activity. Winged bean appears to contain only one type of NR enzyme that is similar to the constitutive NAD(P)H-NR (pH 6.5) enzyme of soybean.

It has been shown that the predominant compound evolved from soybean leaves during the purged *in vivo* NR[3] assay is nitric oxide (NO) with trace amounts of nitrous oxide (N_2O) and nitrogen dioxide (NO_2) also present (4). It was suggested earlier by Harper (5) that the formation of $NO_{(x)}$ was due to an enzymic reaction. Further evidence for an enzymic reaction came from the isolation of a soybean mutant (designated nr_1) whose leaflets lacked both the constitutive NR activity and ability to evolve $NO_{(x)}$ (14, 17). It was also determined (17) that there was joint inheritance of the constitutive NR and $NO_{(x)}$ evolution activities during reciprocal crosses between the wild-type and the nr_1 mutant. Additional support for the association of the constitutive NR and $NO_{(x)}$ evolution activities came from the observation that roots and cell suspension cultures of wild-type soybean plants lack both the constitutive NR and $NO_{(x)}$ evolution activities (6).

It was later determined that there were two forms of constitutive NR in wild-type soybean leaflets, which were designed c_1NR and c_2NR (22). The c_1NR enzyme has a pH optimum of 6.5, prefers NADPH as an electron donor (though NADH may also be used), and has a high K_m for nitrate (5.0 mM). The c_2NR enzyme has a pH optimum of 6.5, prefers NADH as an electron donor, and has a low K_m for nitrate (0.19 mM). The c_1NR and the c_2NR enzymes are designated constitutive since they are present in wild-type soybean leaflets grown on urea or zero N (*i.e.* in the absence of the nitrate inducer). Wild-type soybean leaflets also contain an inducible NR enzyme (iNR) when grown on nitrate. The iNR has a pH optimum of 7.5, prefers NADH as an electron donor, and has a low K_m for nitrate (0.13 mM) (22). The presence of two constitutive NR enzymes was supported by the characterization of two additional soybean mutants (designated LNR-5 and LNR-6) whose leaflets lack the c_2NR enzyme but retain the c_1NR enzyme (20). Leaflets of urea-grown LNR-5 and LNR-6 plants (resulting in the presence of only c_1NR) have been shown to exhibit rates of $NO_{(x)}$ evolution during the purged *in vivo* NR assay that are comparable to rates exhibited by nitrate-grown wild-type plants (all three NR forms present) (20). This indicates that an association exists between the c_1NR enzyme and the ability to evolve $NO_{(x)}$.

The c_1NR enzyme of soybean leaflets is similar to most assimilatory NR enzymes in that it exhibits NADH Cyt *c* reductase activity, $FMNH_2$-NR activity, and reduced MV-NR activity. The c_1NR enzyme is also inhibited by pHMB at the initial site of electron transfer through the enzyme, it is inhibited by KCN, and it presumably contains a molybdenum cofactor at the terminal portion of the enzyme (15). The objectives of this study were to: (a) show that the c_1NR enzyme is responsible for the $NO_{(x)}$ evolution activity; and (b) biochemically characterize the $NO_{(x)}$ evolution activity in terms of its use of alternate electron donors, response to inhibitors, pH optimum, and Michaelis constants for substrates.

[1] Supported by an American Soybean Association research grant, project number 84953.

[2] Present address: Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108.

[3] Abbreviations: NR, nitrate reductase; $NO_{(x)}$, refers collectively to nitric oxide (NO) and nitrogen dioxide (NO_2); $FMNH_2$, reduced flavin mononucleotide; MV, methyl viologen; pHMB, *p*-hydroxymercuribenzoate; FAD, flavin adenine dinucleotide; KCN, potassium cyanide.

MATERIALS AND METHODS

Plant Material, Enzyme Extraction, and Purification. Seeds of wild-type soybean (*Glycine max* [L.] Merr. cv Williams), LNR-5 and LNR-6 soybean mutants, and winged bean (*Psophocarpus tetragonolobus* [L.] DC. cv Lunita) were germinated in either vermiculite or sand and grown as previously described (4).

Extraction of NRs from unifoliolate leaves, followed by an initial purification with Blue Sepharose column chromatography, was performed as described (21) with the following exceptions. Ten milliliters and 100 mL of extraction medium, containing 10 μM FAD and 0.1 M K-phosphate (pH 7.4) instead of Tris-HCl, were used per gram leaf of soybean and winged bean, respectively, and 6 mL of 50 μM NADPH or NADH in washing buffer per gram gel was used for elution of NRs. All eluted fractions were assayed for constitutive NAD(P)H-NR (pH 6.5), constitutive NADH-NR (pH 6.5), inducible NADH-NR (pH 7.5), and $NO_{(x)}$ evolution activities. The NR activities were measured under conditions that had been optimized for each NR form (21, 22). It is important to note that each NR form has a varying degree of activity under the assay conditions optimized for the other NR forms (20). $NO_{(x)}$ evolution activity was determined colorimetrically as nitrite with the system described by Harper (5). The assay mix consisted of one drop of octyl alcohol to prevent frothing, 20 mM K-phosphate (pH 7.5), 20 mM KNO_2, and 0.5 mM NADH (the assay was later optimized to contain 20 mM K-phosphate [pH 6.75] and 5 mM KNO_2). Assays were started by the addition of 1 mL of each eluted fraction to give a final volume of 10 mL. The assay mix (incubated at 30°C) was purged with N_2 for 30 min at a flow rate of 150 mL min^{-1}, and the fritted gas dispersion tube was transferred to a fresh 20 mL volume of trapping solution every 15 min. The Blue Sepharose-purified fractions containing the greatest $NO_{(x)}$ evolution activity were pooled, precipitated with ammonium sulfate, and stored at −18°C. The precipitates were collected when needed by centrifugation, resuspended in washing buffer (minus leupeptin, Pepstatin A, phenylmethylsulfonylfluoride [PMSF], Na-*p*-tosyl-L-lysine chloromethyl ketone [TLCK], and in some cases FAD depending on whether MV or FMN was to be used in subsequent assays), and concentrated in a stirring cell as described by Streit *et al.* (21).

Ion exchange chromatography with a fast protein liquid chromatography (FPLC) Mono Q column (Pharmacia Inc.,[4] Piscataway, NJ) was performed as described by Streit *et al.* (21) except that the flow rate was 1.00 mL min^{-1}. Each fraction eluted from the FPLC Mono Q column was assayed for constitutive NAD(P)H-NR (pH 6.5) and $NO_{(x)}$ evolution activity as described above except that only 0.17 and 0.03 mL of each fraction was used for the $NO_{(x)}$ evolution and NR assays, respectively.

pH Optimum for $NO_{(x)}$ Evolution Activity. Blue Sepharose-purified enzyme extracted from zero N-grown LNR-5 mutants was used as the enzyme source. The assay mix consisted of one drop of octyl alcohol, 50 mM MES, 50 mM K-phosphate, 20 mM KNO_2, and 0.375 mM NAD(P)H; final pH values ranged from 6.25 to 7.25 in 0.25 increments. The assay was started by the addition of 0.1 mL (12.5 μg of protein) of concentrated enzyme to give a final volume of 5 mL. To measure $NO_{(x)}$ formed, the assay mix was purged for 30 min at a flow rate of 150 mL min^{-1}. The fritted gas dispersion tube was transferred to fresh trapping solution every 15 min. Control assays (minus enzyme) were needed to determine the amount of $NO_{(x)}$ that was formed nonenzymically at lower pH values. The A_{540} value obtained in the control assay at each corresponding pH was used to correct the A_{540} value obtained in the actual assay. Both the control and actual assays were replicated three times.

Michaelis Constants for $NO_{(x)}$ Evolution Activity. The enzyme source was the same as decribed for the pH optimum studies. The assay mix used to determine the Michaelis constant (K_m) for nitrite consisted of one drop of octyl alcohol, 20 mM K-phosphate (pH 6.75), 0.375 mM NAD(P)H (saturating), and eight concentrations of nitrite ranging from 0.05 to 5.0 mM. The assay was started by the addition of 0.175 mL (32 μg of protein) of enzyme to give a final volume of 5 mL. $NO_{(x)}$ was measured as described above except that the fritted gas dispersion tube was transferred to 10 mL of fresh trapping solution every 5 min.

Michaelis constants for NADH and NADPH were determined as for nitrite except that the assay mix contained 20 mM nitrite (saturating) and eight concentrations of NADH or NADPH that varied from 7 to 125 μM. The assay was started by the addition of 0.2 mL (16 μg of protein) concentrated enzyme to give a final volume of 5 mL. Apparent K_m values for nitrite, NADH, and NADPH were determined by Hanes-Woolf plots, and each determination was repeated three times.

Partial Activity and Inhibitor Analyses. The Blue Sepharose-purified enzyme was examined for both constitutive NAD(P)H-NR (pH 6.5) and $NO_{(x)}$ evolution activities in the presence ofKCN and pHMB, using either $FMNH_2$, reduced MV, or NADPH as the reductant source. The enzyme was prepared as described above for the pH optimum and kinetic studies except that the resuspension buffer did not contain FAD.

When $FMNH_2$ was used as the reductant source in the presence of KCN, the $NO_{(x)}$ assay medium contained one drop of octyl alcohol, 20 mM K-phosphate (pH 6.75), 1.5 mM FMN, the appropriate concentration of KCN, and 0.2 mL (about 30.5 μg of protein) of concentrated enzyme. The enzyme was prereduced with $FMNH_2$ by adding 0.25 mL of $Na_2S_2O_4$ (0.1 g/25 mL of 10 mM $NaHCO_3$, pH 8.5) and preincubating the assay mix without purging for 5 min at 30°C. The $NO_{(x)}$ assay was then started by the addition of 0.75 mL of a KNO_2 solution to give a final concentration of 5 mM in a final volume of 5 mL. $NO_{(x)}$ was detected as previously described. The fritted gas dispersion tube was transferred to fresh trapping solution every 15 min.

When $FMNH_2$ was used as the reductant source in the presence of pHMB, the $NO_{(x)}$ assay mix was the same as used in the KCN inhibition studies except that 0.7 mL of a solution containing the appropriate concentration of pHMB dissolved in 14 mM NaOH and 10 mM $NaHCO_3$ (pH 8.5) replaced the KCN. In addition, the enzyme was not prereduced, the assay was started by the addition of $Na_2S_2O_4$, and the assay time was only 15 min. MV- and NADPH-$NO_{(x)}$ assays were conducted the same as the $FMNH_2$-$NO_{(x)}$ assays except that where appropriate 1.0 mM MV MV replaced the FMN and 0.375 mM NADPH replaced both the FMN and $Na_2S_2O_4$. In all experiments, the A_{540} value of the trapping solution, following the actual $NO_{(x)}$ assay, was corrected for the A_{540} value obtained from a minus enzyme control assay.

To measure $FMNH_2$-NR activity in the presence of KCN, the assay mix consisted of 20 mM K-phosphate (pH 6.5), 1.5 mM FMN, the appropriate concentration of KCN, and 0.04 mL (about 6.1 μg of protein) of concentrated enzyme. The enzyme was prereduced as before with 0.05 mL of $Na_2S_2O_4$ (0.1 g/25 mL of 10 mM $NaHCO_3$, pH 8.5) and the assay was started by the addition of 0.15 mL of a KNO_3 solution to give a final concentration of 80 mM in a final volume of 1 mL. The 15-min assay was terminated by vortexing the assay mix for 20 s. Color was developed by the addition of 1 mL of sulfanilamide (10 g/L of 1.5 N HCl) followed by 1 mL of *n*-1-napthylethylenediamine diHCl (0.2 g/L of H_2O).

To measure $FMNH_2$-NR activity in the presence of pHMB, the NR assay mix was the same as used in the KCN inhibition studies except that 0.14 mL of a solution containing the appro-

[4] Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the vendor or product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other vendors or products that may also be suitable.

priate concentration of pHMB dissolved in 14 mM NaOH and 10 mM $NaHCO_3$ (pH 8.5) replaced the KCN, the enzyme was not prereduced, and the assay was started by the addition of $Na_2S_2O_4$. The MV- and NADPH-NR assays were conducted the same as the $FMNH_2$-NR assays except that 1.0 mM MV and 0.375 mM NADPH replaced the appropriate reagents. The MV-NR assays were terminated by vortexing as with the $FMNH_2$-NR assays, and the NADPH-NR assays were terminated by the addition of 1 mL of sulfanilamide (10 g/L of 1.5 N HCl), and color was developed as described above.

Protein. Protein was determined by the Bradford method (2) with BSA as a protein standard.

RESULTS

Elution Profiles. Both the constitutive NAD(P)H-NR (pH 6.5) (c_1NR) and $NO_{(x)}$ evolution activities were eluted from Blue Sepharose with NADPH when either nitrate- or zero N-grown wild-type soybean (cv Williams) was used as the enzyme source (Fig. 1, A and B). The elution profile of the $NO_{(x)}$ evolution activity coincided with the elution profile of the c_1NR activity. NADH eluted both the constitutive NADH-NR (pH 6.5) (c_2NR) and the inducible NADH-NR (pH 7.5) (iNR) activity from nitrate-grown Williams and only the c_2NR activity from zero N-grown Williams (Fig. 1, A and B). None of the NADH-eluted fractions were found to express $NO_{(x)}$ evolution activity.

When zero N-grown LNR-5 and LNR-6 soybean mutants were used as the enzyme source, both the c_1NR and the $NO_{(x)}$ evolution activities were again eluted from Blue Sepharose with NADPH and the activity profiles coincided (Fig. 2, A and B). The peaks of activity representing c_2NR and iNR in the NADPH eluted fractions (Fig. 2, A and B) were not due to the presence of these enzymes, but rather to the presence of the c_1NR enzyme which has some measurable activity under the assay conditions optimized for the other two NR forms (20). NADH did not elute any NR activity from the blue Sepharose since the LNR-5 and LNR-6 mutants have been characterized as lacking the c_2NR enzyme (20), and the iNR was eliminated due to growth on zero N.

Elution profiles from Blue Sepharose loaded with extracts from nitrate- and zero N-grown winged bean are shown in Figure 3, A and B. All of the NR and $NO_{(x)}$ evolution activity was eluted with NADPH regardless of whether nitrate- or zero N-grown leaves were used as the starting material. The NR activity eluted with NADPH had maximum activity with the assay conditions that had been optimized for soybean c_1NR. The fractions containing NR activity were the same as those containing $NO_{(x)}$ evolution activity. The activities of both the NR and $NO_{(x)}$ assays from winged bean were severalfold higher than the activities obtained from soybean, even though the amount of soybean leaf tissues used for extraction was three times greater than the amount of winged bean leaf tissue.

The lack of c_2NR and iNR in urea- or zero N-grown LNR-5 and LNR-6 mutant plants eliminated the possibility that a small amount of either of these enzymes might be eluted with NADPH during Blue Sepharose chromatography and cross-contaminate the fractions containing c_1NR and $NO_{(x)}$ evolution activities. Therefore, unifoliolate leaves from these two sources (particularly LNR-5 due to better expansion of unifoliolate leaves) provided good starting material for further purification. In addition, the calculations of recovery and magnitude of purification are more accurate since the initial activity expressed in the crude extracts

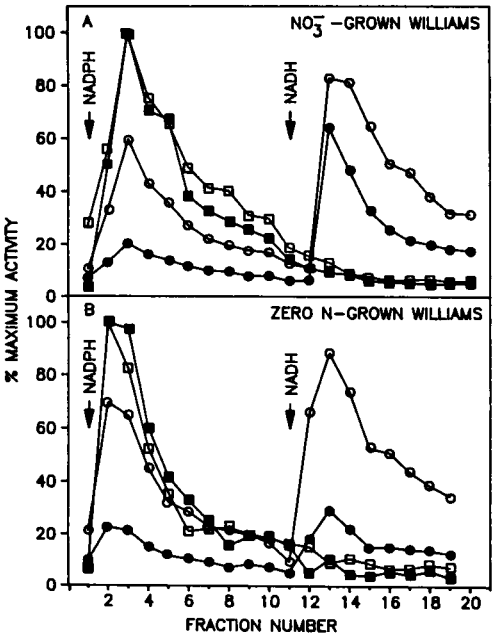

FIG. 1. Elution profiles of c_1NR (■—■), c_2NR (○—○), iNR (●—●), and $NO_{(x)}$ evolution (□—□) activities from Blue Sepharose columns loaded with extracts from unifoliolate leaves of (A) nitrate-grown or (B) zero N-grown Williams soybean. Enzymes were extracted from 30 g of leaves in 300 mL of extraction buffer that consisted of 100 mM K-phosphate (pH 7.4), 1 mM EDTA, 10 mM L-cysteine, 10 μM FAD, and 0.5% casein. The extract was mixed with 10 g of Blue Sepharose, and activities were sequentially eluted with 60 mL of NADPH (0.05 mM) followed by 60 mL of NADH (0.05 mM) in washing buffer that consisted of 100 mM K-phosphate (pH 7.8), 1 mM EDTA, 1 mM DTT, and 10 μM FAD. Enzyme activities were assayed as described under "Materials and Methods." Maximum activities were: A, 20.9 nmol min^{-1} mL^{-1} for NR activity and 12.8 nmol min^{-1} mL^{-1} for $NO_{(x)}$ evolution activity; B, 14.5 nmol min^{-1} mL^{-1} for NR activity and 6.01 nmol min^{-1} mL^{-1} for $NO_{(x)}$ evolution activity.

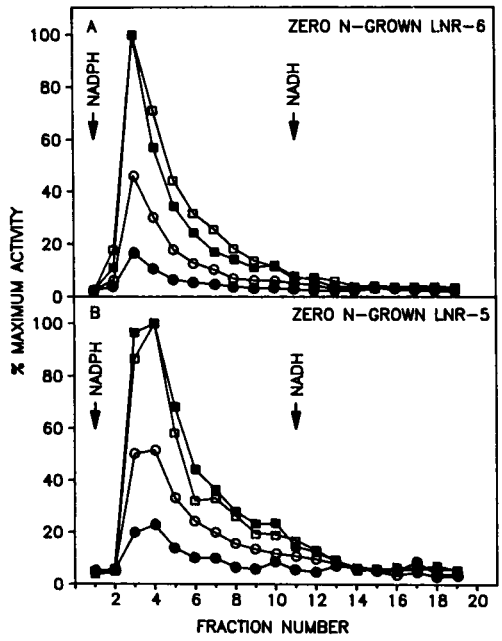

FIG. 2. Elution profiles of c_1NR (■—■), c_2NR (○—○), iNR (●—●), and $NO_{(x)}$ evolution (□—□) activities from Blue Sepharose columns loaded with extracts from unifoliolate leaves of (A) zero N-grown LNR-6 or (B) LNR-5. Blue Sepharose chromatography was as described in Figure 1 legend. Maximum activities were: A, 22.0 nmol min^{-1} mL^{-1} for NR activity and 10.2 nmol min^{-1} mL^{-1} for $NO_{(x)}$ evolution activity; B, 10.31 nmol min^{-1} mL^{-1} for NR activity and 4.32 nmol min^{-1} mL^{-1} for $NO_{(x)}$ evolution activity.

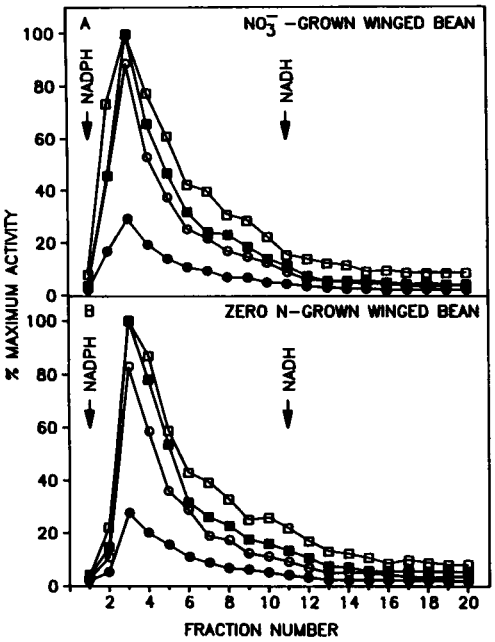

FIG. 3. Elution profiles of winged bean (cv Lunita) NR and $NO_{(x)}$ evolution activities from Blue Sepharose columns loaded with extracts from (A) nitrate-grown or (B) zero N-grown plants. Activity was measured with assay conditions that had been optimized for soybean c_1NR (■—■), c_2NR (○—○), iNR (●—●), and $NO_{(x)}$ evolution activity (□—□). Blue Sepharose chromatography was as described in Figure 1 legend except that only 3 g of leaf tissue were used. Maximum activities were: A, 212.7 nmol min^{-1} mL^{-1} for the NR activity and 29.7 nmol min^{-1} mL^{-1} for the $NO_{(x)}$ evolution activity; B, 80 nmol min^{-1} mL^{-1} for the NR activity and 15.1 nmol min^{-1} mL^{-1} for the $NO_{(x)}$ evolution activity.

represents only the c_1NR activity and not a mixture of the isozymes.

An FPLC-Mono Q anion exchange column was used following Blue Sepharose chromatography in an attempt to separate the c_1NR activity from the $NO_{(x)}$ evolution activity extracted from urea-grown LNR-5 mutant plant leaves. Following this step, the elution profiles for both the c_1NR and the $NO_{(x)}$ evolution activities still coincided (Fig. 4), indicating that the two activities were not separated. The two-step purification described resulted in a greater than 2500- and 2900-fold purification with a 4.5 and 5.5% recovery when based on the c_1NR and the $NO_{(x)}$ evolution activities, respectively (Table I).

pH Optimum and K_m Values for $NO_{(x)}$ Evolution Activity. The determination of the pH optimum for *in vitro* $NO_{(x)}$ evolution activity was confounded by the nonenzymic conversion of nitrite to gaseous nitrogen compounds under conditions of low pH. Control assays run in the absence of enzyme revealed that substantial amounts of nitrogenous compound detected by the preoxidizer/Greiss-Saltzman assay were produced as the pH of the assay medium was decreased below 6.75. Therefore, the amount of nitrogenous compound produced during control (minus enzyme) assays was subtracted from the amount of $NO_{(x)}$ produced during the actual enzyme assay. The results, corrected for nonenzymic NO_2^- reduction, are depicted in Figure 5. The pH optimum for the *in vitro* enzymic $NO_{(x)}$ evolution activity was 6.75 regardless of whether NADPH or NADH was used as the reductant source (Fig. 5). The pH optimum for the c_1NR nitrate reductase activity was previously determined to be 6.5 (3, 22).

The apparent K_m for nitrite during $NO_{(x)}$ evolutin activity was the same regardless of whether excess NADPH or NADH served as the electron donor (Table II). The apparent K_m values for either NADPH or NADH in the presence of excess nitrite were also very nearly the same (Table II).

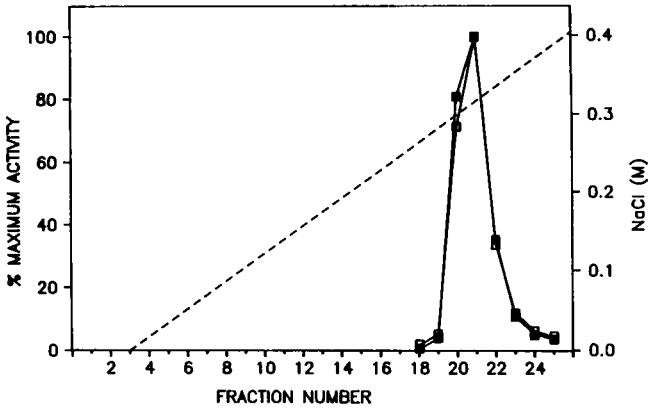

FIG. 4. Elution profiles of c_1NR (■—■) and $NO_{(x)}$ evolution (□—□) activities following purification on an FPLC Mono Q anion exchange column. The most active fractions containing c_1NR and $NO_{(x)}$ evolution activity from Blue Sepharose columns loaded with extracts from urea-grown LNR-5 mutant plants provided the enzyme source. Protein was eluted from the column with a linear gradient of NaCl (– – –). The flow rate was 1.0 mL min^{-1}, and the elution volume was 25 mL. Maximum activities were: 285.3 nmol min^{-1} mL^{-1} for the c_1NR activity and 144.9 nmol min^{-1} mL^{-1} for the $NO_{(x)}$ evolution activity. See "Materials and Methods" for further experimental details.

Comparison of Electron Donors and Inhibitor Response. The Blue Sepharose-purified enzyme had the ability to use $FMNH_2$ and reduced MV as electron donors, in addition to NAD(P)H, for the $NO_{(x)}$ evolution activity (Tables III and IV in the absence of inhibitor). As determined before by Nelson *et al.* (15), these same electron donors will support c_1NR activity (Tables III and IV). However, as seen in Tables III and IV in the absence of inhibitor, the efficiency of each electron donor was different for each activity.

The NADPH-, $FMNH_2$- and reduced MV-c_1NR and the NADPH-, $FMNH_2$-, and reduced MV-$NO_{(x)}$ evolution activities were all inhibited by increasing concentrations of KCN (Table III). In each case, the increasing concentrations of KCN inhibited the c_1NR and $NO_{(x)}$ evolution activities in a similar, though not identical, manner.

The NADPH-c_1NR and NADPH-$NO_{(x)}$ evolution activities were also both inhibited by increasing concentrations of pHMB; however, the NADPH-$NO_{(x)}$ evolution activity appeared to be more sensitive to this inhibitor than the NADPH-c_1NR activity (Table IV). The $FMNH_2$- and MV-c_1NR and $FMNH_2$- and MV-$NO_{(x)}$ evolution activities were much less sensitive to inhibition by pHMB (Table IV).

DISCUSSION

The inability to separate the $NO_{(x)}$ evolution activity from the c_1NR activity, using Blue Sepharose affinity chromatography followed by FPLC-anion exchange chromatography, provided strong evidence that the two activities were associated with the same enzyme. At each step of the purification, the magnitude of purification and percent recovery for both activities were very similar (Table I). This two-step purification procedure resulted in a nearly homogenous preparation of the c_1NR enzyme (21). Therefore, it is unlikely that another enzyme with $NO_{(x)}$ evolution activity is copurifying with the c_1NR enzyme.

The leaf tissue of most higher plants contains a single, nitrate-inducible, NADH-preferring NR with a pH optimum of 7.5 (1).

Table I. *Purification of c_1NR and $NO_{(x)}$ Evolution Activity from Urea-Grown LNR-5 Mutant Plants*
The data are based either on the c_1NR (top) or the $NO_{(x)}$ (bottom) activities.

Fraction	Total		Specific Activity	Purification	Recovery
	Activity	Protein			
	μmol min^{-1}	*mg*	*μmol min^{-1} mg^{-1}*	*-fold*	*%*
Based on c_1NR activity					
Centrifuged homogenate	13.72	2624	0.005	1	100
Blue Sepharose pooled peaks	2.04	1.87	1.091	218	15.4
FPLC-Mono Q pooled peaks	0.617	0.048	12.85	2571	4.5
Based on $NO_{(x)}$ evolution activity					
Centrifuged homogenate	4.91	2624	0.002	1	100
Blue Sepharose pooled peaks	0.749	1.87	0.401	211	15.2
FPLC-Mono Q pooled peaks	0.272	0.048	5.67	2982	5.5

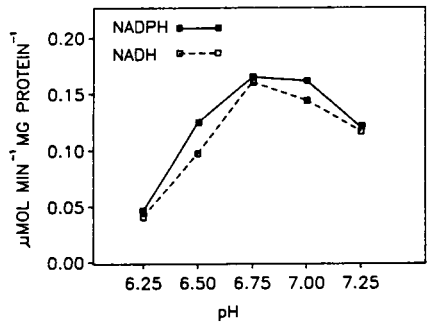

FIG. 5. pH optimum for NADPH- or NADH-mediated $NO_{(x)}$ evolution activity. The assays were conducted as described under "Materials and Methods." Each data point represents the difference between the actual enzyme assays and control (minus enzyme) assays. Control assays were needed at the lower pH values to determine the amount of nonenzymically produced $NO_{(x)}$. Each experiment was repeated three times.

Table II. *Michaelis Constants (Nitrite and Pyridine Nucleotide) of Blue Sepharose Purified c_1NR during $NO_{(x)}$ Evolution Activity*
Assays were performed as described in "Materials and Methods."

Substrate	K_m
Nitrite	
With NADPH	0.49 mM
With NADH	0.49 mM
NADPH	7.2 μM
NADH	7.4 μM

However, in addition to the inducible enzyme, soybean also contains two constitutive NR enzymes designated c_1NR and c_2NR (22). The elution profiles obtained from Blue Sepharose columns loaded with extracts from winged bean revealed that all the NR and $NO_{(x)}$ evolution activity was eluted with NADPH. The NR activity was highest under assay conditions that have been optimized for the soybean c_1NR enzyme, and the elution profiles for both nitrate- and zero N-grown winged bean were identical. Though the physiological and biochemical characteristics of winged bean NR should be further investigated, it appears that this legume contains only one type of NR that is similar to the c_1NR of soybean. Stewart and Orebamjo (19) have shown that the tropical legume *Erythrina senegalensis* contains only the nonspecific NAD(P)H-NR enzyme, which may be the same type of enzyme found in winged bean.

The c_1NR enzyme of soybean appears to contain the same components and partial activities, with the exception of the $NO_{(x)}$ evolution activity, as most other higher plant assimilatory NR enzymes (15). Typically, the NAD(P)H-, MV-, and $FMNH_2$-mediated nitrate reduction is inhibited by cyanide, whereas the NAD(P)H-dehydrogenase activity is not affected. Reduced viologen dyes, $FMNH_2$, and reduced pyridine nucleotides can mediate NR activity; however, only NAD(P)H can mediate the dehydrogenase activity. Mercurial compounds (pHMB) inhibit the NAD(P)H-dehydrogenase and NAD(P)H-NR activities while the MV- and $FMNH_2$-NR activities are relatively insensitive. Based on the partial activities and the pattern of inhibition, it was determined that NAD(P)H donates electrons to the front portion of the enzyme while MV and $FMNH_2$ donate electrons to the terminal cofactor-containing portion of the enzyme (see reviews in 1 and 8). In this study it was found that MV, $FMNH_2$, and NAD(P)H would mediate the $NO_{(x)}$ evolution activity of the c_1NR enzyme and that these activities were all inhibited by increasing concentrations of cyanide. This indicates that the reduction of nitrite to $NO_{(x)}$ takes place at the terminal molybdenum-containing portion of the enzyme. This was further supported by the observation that the NAD(P)H-$NO_{(x)}$ evolution activity, but not the MV- or $FMNH_2$-$NO_{(x)}$ evolution activities, was inhibited by increasing concentrations of pHMB. The highest c_1NR activity in the absence of inhibitor was obtained with $FMNH_2$ followed by MV and NADPH, but the highest $NO_{(x)}$ evolution activity was obtained with NADPH followed by $FMNH_2$ and MV (Tables III and IV). This discrepancy might be explained by the nonenzymic conversion of NO to NH_2OH in the presence of dithionite, which was used to reduce the MV and FMN. This phenomenon has been documented during studies on bacterial denitrification (13, 23). NH_2OH was not detected by the preoxidizer/Greiss-Saltzman method used in this study (data not shown); therefore, the values of $NO_{(x)}$ produced when dithionite reduced MV or FMN was used as electron donors may be underestimated.

It is tempting to make comparisons between the $NO_{(x)}$ evolution activity of the c_1NR enzyme isolated from soybean and the dissimilatory nitrite reductase isolated from denitrifying bacteria. Nitrite reductases of denitrifying bacteria exist either as a cytochrome *cd* or a copper enzyme. The cytochrome *cd*-type has been found in *Pseudomonas aeruginosa* (24), *Micrococcus deni-*

Table III. *Effect of KCN on NADPH-, $FMNH_2$-, and MV-c_1NR and NADPH-, $FMNH_2$-, and MV-$NO_{(x)}$ Evolution Activities*

Assays were performed as described in "Materials and Methods." Values listed are the means of the three replications ± SD.

Electron Donor	Concentration of KCN	Initial Activity		Percent of Initial Activity	
		c_1NR activity	$NO_{(x)}$ evolution activity	c_1NR activity	$NO_{(x)}$ evolution activity
	mM	μmol min^{-1} mg^{-1} protein		%	
NADPH	0.00	0.100 ± 0.008	0.112 ± 0.011	100 ± 5.2	100 ± 9.5
	0.01			105.5 ± 2.5	92.7 ± 2.7
	0.10			82.5 ± 3.7	74.8 ± 5.0
	1.00			28.6 ± 2.0	21.8 ± 0.9
$FMNH_2$	0.00	0.231 ± 0.008	0.061 ± 0.002	100 ± 3.2	100 ± 3.1
	0.01			86.7 ± 4.8	70.6 ± 4.7
	0.10			42.9 ± 0.6	33.3 ± 3.6
	1.00			1.2 ± 0.3	4.7 ± 2.2
MV	0.00	0.207 ± 0.012	0.016 ± 0.001	100 ± 5.6	100 ± 8.0
	0.01			91.9 ± 2.1	86.8 ± 5.6
	0.10			43.2 ± 1.7	32.3 ± 7.6
	1.00			3.7 ± 0.3	3.2 ± 0.0

Table IV. *Effect of pHMB on NADPH-, $FMNH_2$-, and MV-c_1NR and NADPH-, $FMNH_2$-, and MV-$NO_{(x)}$ Evolution Activities*

Assays were performed as described in "Materials and Methods." Values listed are the means of three replications ± SD.

Electron Donor	Concentration of pHMB	Initial Activity		Percent Initial Activity	
		c_1NR activity	$NO_{(x)}$ evolution activity	c_1NR activity	$NO_{(x)}$ evolution activity
	mM	μmol min^{-1} mg^{-1} protein		%	
NADPH	0.00	0.095 ± 0.007	0.209 ± 0.059	100 ± 4.2	100 ± 12.3
	0.05			67.1 ± 4.4	58.9 ± 5.2
	0.10			54.3 ± 1.1	42.0 ± 6.6
	0.15			42.9 ± 1.6	21.7 ± 1.0
$FMNH_2$	0.00	0.483 ± 0.020	0.058 ± 0.009	100 ± 4.0	100 ± 5.1
	0.05			95.3 ± 2.4	88.1 ± 3.5
	0.10			96.3 ± 4.9	111 ± 12.8
	0.15			79.0 ± 5.1	90.8 ± 3.9
MV	0.00	0.371 ± 0.009	0.034 ± 0.003	100 ± 2.5	100 ± 6.5
	0.05			103.0 ± 2.9	101.7 ± 8.6
	0.10			106.8 ± 6.2	112.5 ± 4.2
	0.15			98.1 ± 4.5	91.0 ± 8.8

trificans (16), *Alcaligenes faecalis* (9), and *Pseudomonas stutzeri* (12). The copper-containing enzyme has been found in Alcaligenes sp. (11), *Achromobacter cycloclastes* (10), and *Rhodopseudomonas sphaeroides* (18). No reports of a molybdenum-containing dissimilatory nitrite reductase could be found. The nitrite reductase isolated from *Achromobacter cycloclastes* has a K_m for nitrite of 0.5 mM (10), which is similar to the K_m for nitrite of the c_1NR enzyme from soybean during $NO_{(x)}$ evolution activity (Table II). It is interesting that the c_1NR enzyme has a higher affinity for nitrite than for nitrate. However, under normal conditions of plant growth nitrite rarely accumulates in plant tissues. Therefore, it is unlikely that substantial amounts of $NO_{(x)}$ are evolved from intact soybean under ambient conditions.

The $NO_{(x)}$ evolution activity appears to be a unique biochemical marker for the soybean c_1NR enzyme or an NR enzyme similar to the c_1NR enzyme as found in winged bean. $NO_{(x)}$ assays *in vivo* or *in vitro* could provide a means of screening various plant species for the presence of a c_1NR-type enzyme. This could be of taxonomic significance since the presence of a constitutive-NR enzyme is unique among higher plants (7), and $NO_{(x)}$ evolution in higher plants appears to be limited to the Phaseoleae tribe of the family Leguminosae: (Papilionoideae) (4).

LITERATURE CITED

1. BEEVERS L, RH HAGEMAN 1983 Uptake and reduction of nitrate: bacteria and higher plants. *In* A Lauchli, RL Bieleski, eds, Inorganic Plant Nutrition, Encyclopedia of Plant Physiology, New Series, Vol 15A. Springer Verlag, New York, pp 351–375
2. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
3. CAMPBELL WH 1976 Separation of soybean leaf nitrate reductases by affinity chromatography. Plant Sci Lett 7: 239–247
4. DEAN JV, JE HARPER 1986 Nitric oxide and nitrous oxide production by soybean and winged bean during the *in vivo* nitrate reductase assay. Plant Physiol 82: 718–723
5. HARPER JE 1981 Evolution of nitrogen oxide(s) during *in vivo* nitrate reductase assay of soybean leaves. Plant Physiol 68: 1488–1493
6. HARPER JE, RS NELSON, L STREIT 1985 Nitrate metabolism of soybean-

physiology and genetics. *In* RW Shibles, ed, World Soybean Research Conference III: Proceedings. Westview Press, Boulder, CO, pp 476–483
7. HARPER JE 1987 Nitrogen metabolism. *In* JR Wilcox, ed, Soybeans: Improvement, Production, and Uses, 2nd ed. Agronomy Monograph No. 16, Madison, WI, pp 497–533
8. HEWITT EJ, BA NOTTON 1980 Nitrate reductase systems in eukaryotic and prokaryotic organisms. *In* MP Coughlan, ed, Molybdenum and Molybdenum-containing Enzymes. Pergamon Press, New York, pp 273–325
9. IWASAKI H, T MATSUBARA 1971 Cytochrome c-557 (551) and cytochrome *cd* of *Alcaligenes faecalis*. J Biochem 69: 847–857
10. IWASAKI H, T MATSUBARA 1972 A nitrite reductase from *Achromobacter cycloclastes*. J Biochem 71: 645–652
11. IWASAKI H, S SHIDARA, H SUZUKI, T MORI 1963 Studies on denitrification. VII. Further purification and properties of denitrifying enzyme. J Biochem 53: 299–303
12. KODAMA T 1970 Effects of growth conditions on formation of cytochrome system of a denitrifying bacterium, *Pseudomonas stutzeri*. Plant Cell Physiol 11: 231–239
13. MASUKO M, H IWASAKI 1984 Activation of nitrite reductase in a cell-free system from the denitrifier *Alcaligenes* sp. by freeze-thawing. Plant Cell Physiol 25: 439–446
14. NELSON RS, SA RYAN, JE HARPER 1983 Soybean mutants lacking constitutive nitrate reductase activity. I. Selection and initial plant characterization. Plant Physiol 72: 503–509
15. NELSON RS, L STREIT, JE HARPER 1986 Nitrate reductases from wild-type and nr_1-mutant soybean (*Glycine max* [L.] Merr.) leaves. II. Partial activity, inhibitor, and complementation analyses. Plant Physiol 80: 72–76
16. NEWTON N 1969 The two-haem nitrite reductase of *Micrococcus denitrificans*. Biochim Biophys Acta 185: 316–331
17. RYAN SA, RS NELSON, JE HARPER 1983 Soybean mutants lacking constitutive nitrate reductase activity. II. Nitrogen assimilation, chlorate resistance, and inheritance. Plant Physiol 72: 510–514
18. SAWADA E, T SATOH, H KITAMURA 1978 Purification and properties of a dissimilatory nitrite reductase of a denitrifying phototrophic bacterium. Plant Cell Physiol 19: 1339–1351
19. STEWART GR, TO OREBAMJO 1979 Some unusual characteristics of nitrate reduction in *Erythrina senegalensis* DC. New Phytol 83: 311–319
20. STREIT L, JE HARPER 1986 Biochemical characterization of soybean mutants lacking constitutive NADH:nitrate reductase. Plant Physiol 81: 593–596
21. STREIT L, BA MARTIN, JE HARPER 1987 A method for the separation and partial purification of the three forms of nitrate reductase present in wild-type soybean leaves. Plant Physiol 84: 654–657
22. STREIT L, RS NELSON, JE HARPER 1985 Nitrate reductases from wild-type and nr_1-mutant soybean (*Glycine max* [L.] Merr.) leaves. I. Purification, kinetics, and physical properties. Plant Physiol 78: 80–84
23. URATA K, T SATOH 1985 Mechanism of nitrite reduction to nitrous oxide in a photodenitrifier, *Rhodopseudomonas sephaeroides* forma sp. *denitrificans*. Biochim Biophys Acta 841: 201–207
24. YAMANAKA T, K OKUNUKI 1963 Crystalline *Pseudomonas* cytochrome oxidase. I. Enzymic properties with special reference to the biological specificity. Biochim Biophys Acta 67: 379–393