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Nitrate Reductases from Wild-Type and nr_1-Mutant Soybean (*Glycine max* [L.] Merr.) Leaves[1]

II. PARTIAL ACTIVITY, INHIBITOR, AND COMPLEMENTATION ANALYSES

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RICHARD S. NELSON[2], LUC STREIT, AND JAMES E. HARPER*
Department of Agronomy (R.S.N., L.S.) *and United States Department of Agriculture, Agricultural Research Service* (J.E.H.), *University of Illinois, Urbana, Illinois 61801*

ABSTRACT

Soybean (*Glycine max* [L.] Merr.) leaves have been shown to contain three forms of nitrate reductase (NR). Two of the forms, which are present in leaves of wild-type (cv. Williams) plants grown in the absence of NO_3^-, are termed constitutive and designated c_1NR and c_2NR. The third form, which is present in NO_3^--grown mutant (nr_1) plants lacking the constitutive forms, is termed inducible and designated iNR. Samples of c_1NR, c_2NR, and iNR obtained from appropriately treated plants were analyzed for the presence of partial activities, response to inhibitors, and ability to complement a barley NR which lacks the molybdenum cofactor (MoCo) but is otherwise active.

The three forms were similar to most assimilatory NR enzymes in that they (a) exhibited NADH-cytochrome *c* reductase, reduced flavin mononucleotide-NR, and reduced methyl viologen-NR partial activities; (b) were inhibited by *p*-hydroxymercuribenzoate at the site of initial electron transport through each enzyme; (c) were more inhibited by CN^- in their reduced enzyme state as compared with their oxidized state; and (d) complemented a MoCo-defective NR (*e.g.* contained cofactors with characteristics similar to the MoCo found in barley NR and commercial xanthine oxidase). However, among themselves, they showed dissimilarities in their response to treatment with HCO_3^- and CN^-, and in their absolute ability to complement the barley NR. The site of effect for these treatments was the terminal cofactor-containing portion of each enzyme. This indicated that, although a terminal cofactor (presumably a MoCo) was present in each form, structural or conformational differences existed in the terminal cofactor-protein complex of each form.

Assimilatory NR[3] from higher plants has been characterized as a flavin- and heme-containing molybdoenzyme which catalyzes several partial reactions (NADH-Cyt *c* reductase, reduced flavin-NR, MV°-NR) in addition to the NAD(P)H-NR reaction (7, 9). The NADH-Cyt *c* reductase (diaphorase) activity, associated with the site of initial electron transfer through the enzyme, is inhibited by mercurial agents (pCMB, pHMB) whereas cyanide (CN^-) inhibits the terminal reduced flavin- and MV°-NR activities associated with the MoCo (7, 9, 15). The MoCo is a molybdenum-molybdopterin complex (16).

Analysis of soybean NR began with Evans and Nason (8) and Nicholas and Nason (25), who showed that pHMB and CN^- inhibited activity and that a flavin and Mo were NR constituents in the partially purified fractions. Jolly *et al.* (17) and Campbell (5), later separated and biochemically characterized two NR forms thus making it difficult to interpret the older experiments where isozymes were not identified. The NAD(P)H:NR was much less sensitive to CN^- inhibition than the NADH:NR (17). During this period, Harper (11) and Lahav *et al.* (19) demonstrated the presence of NR activity in leaves of soybean grown without NO_3^-. A mutant plant (nr_1), which lacked this non-NO_3^--induced or cNR activity but retained NO_3^--induced activity, was later isolated (22, 28). The cNR and iNR activities were shown to be under different genetic control and therefore isozymes (for review, see Harper *et al.* [14]). Tungstate, an analog of molybdate, was shown to inhibit iNR but not cNR activity (2, 13, 18). This finding caused Aslam (2) to suggest that Mo may not be required for cNR activity.

The relationship of the biochemically defined (NADPH:NR and NADH:NR) and the physiologically and genetically defined (cNR and iNR) activities remained obscure until recently. Streit *et al.* (32) demonstrated the presence of three NR forms; one inducible (iNR) and two constitutive (c_1NR, c_2NR) forms. Robin *et al.* (27) and Streit *et al.* (32) have immunologically and kinetically shown c_1NR to be identical to NADPH:NR. The c_2NR had characteristics similar to the cNR activity described by Nelson *et al.* (23), while iNR was similar to the NO_3^--inducible NADH:NR found in all higher plants (32). The relationship between NADPH:NR and c_1NR linked the CN^- and tungstate inhibition observations and further suggested that the MoCo of this form was altered. In addition, since c_2NR was the form characterized in crude extracts (18, 23, 32), and crude extracts were assayed for tungstate inhibition (2), the presence of a normal MoCo in this form was also questionable.

The purpose of this study was to further characterize the three purified soybean NR forms in terms of partial activities and response to pHMB and CN^-. In addition, the state of the terminal moiety (present, absent, or altered MoCo) of each form was determined by inhibitor and complementation analyses.

MATERIALS AND METHODS

Soybean Plant Growth, Enzyme Extraction, and Purification. Procedures were as described by Streit *et al.* (32) except that the

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[2] Present address: Department of Biology, Washington University, Box 1137, St. Louis, MO 63130.

[3] Abbreviations: NR, nitrate reductase; MV°, reduced methyl viologen; pCMB, *p*-chloromercuribenzoic acid; pHMB, *p*-hydroxymercuribenzoate; MoCo, molybdenum cofactor; cNR, constitutive nitrate reductase; iNR, inducible nitrate reductase; $FMNH_2$ reduced flavin mononucleotide; FAD, flavin adenine dinucleotide; PMS, phenazine methosulfate.

Table I. *Reaction Mix Composition for NAD(P)H-NR Assays, for Each Purified Soybean NR Form, Run in the Presence of pHMB*

Further details are given in "Materials and Methods."

Volume of Stock Solution used in Assay	Stock Solution Constituents for Each NR Form		
μl	c_1NR	c_2NR	*iNR*
300	125 mM K^+-phosphate (pH 6.5), 33 mM KNO_3	125 mM K^+-phosphate (pH 6.5), 17 mM KNO_3	125 mM K^+-phosphate (pH 7.5), 17 mM KNO_3
62.5	2 mg NADPH/ml of 10 mM $NaHCO_3$ (pH 8.5)	2 mg NADH/ml of 10 mM $NaHCO_3$ (pH 8.5)	2 mg NADH/ml of 10 mM $NaHCO_3$ (pH 8.5)
62.5	Inhibitor in 14 mM NaOH and 10 mM $NaHCO_3$ (pH 8.5)		
50	Enzyme diluted (10×) with solution containing 100 mM phosphate, 1 mM EDTA, 1 mM DTT, 7 μM FAD, final pH 7.4		

extraction mixture casein concentration was 0.5% (w/v) and protease inhibitors (0.4 mg leupeptin, 4 mg Na-*p*-tosyl-L-lysine chloromethylketone [TLCK], 0.4 mg pepstatin A, and 70 mg phenylmethylsulfonylfluoride [PMSF] per 400 ml) were added to the NAD(P)H-containing buffers for elution of enzyme from blue sepharose. Where pertinent, the extent of purification and specific activities (one unit = 1 μmol product·min^{-1}) for the NR forms are noted in figure and table legends.

Partial Activity Analyses. The NAD(P)H-NR activities were measured under the optimal conditions for each NR form as described by Streit *et al.* (32). The $FMNH_2$-NR activities were measured as described by Nelson *et al.* (23) modified as follows. The assay medium contained 25 mM K^+-phosphate and NO_3^- at the pH and concentration, respectively, determined to be optimal for each NR form during NAD(P)H-NR assays (32). In addition, the assay medium contained 0.5 mM $NaHCO_3$ (pH 8.5) in place of K_2CO_3, 0.1 ml enzyme, and the reaction was started by addition of 0.05 ml $Na_2S_2O_4$ (0.697 g/50 ml of 10 mM $NaHCO_3$, pH 8.5). The final assay volume was 1 ml and, after oxidation of the $FMNH_2$, 0.1 ml of zinc acetate was added. The NAD(P)H-Cyt *c* reductase (diaphorase) activities were measured as described by Wray and Filner (33) except that the assay mixture pH was 7.0 and the final assay volume was 0.5 ml. Activity was calculated using the difference extinction coefficient (20.5 mM^{-1} cm^{-1} at 550 nm) noted by Nelson *et al.* (23). The MV^o-NR activities were measured by the same procedure described above for $FMNH_2$-NR activities with the following modifications. The FMN was replaced with 0.33 mM MV and the $Na_2S_2O_4$ stock solution was 0.5 g/50 ml of 10 mM $NaHCO_3$ (pH 8.5).

Inhibitor Analyses. The NAD(P)H-NR assays performed in the presence of pHMB, were run under the conditions described in Table I. For determination of activities in the presence of 10.5 μM DTT, stored enzyme preparations containing 1 mM DTT were diluted with solution lacking DTT. The reaction was terminated after 15 min by addition of 0.1 ml of 1 M zinc acetate. After centrifugation, 375 μl of supernatant was reacted with 125 μl of 78 μM PMS for 30 min. Nitrite was determined by modification of the procedure of Hageman and Hucklesby (10) with sequential additions of 250 μl 0.5% (w/v) sulfanilic acid in 0.75 N HCl and 250 μl 0.01% (w/v) *N*-1-napthylethylenediamine dihydrochloride. The above procedure was modified as follows for determination of the NAD(P)H-NR and MV^o-NR activities in the presence of 50 μM pHMB. Enzyme was diluted with solution containing 5 mM phosphate and no DTT. The KNO_3-containing stock solution for c_1NR assays contained 170 mM KNO_3. For the MV^o-NR assays, NAD(P)H was replaced with 37.5 μl MV (4 mg/ml deionized H_2O) and 25 μl of $Na_2S_2O_4$ (13.2 mg/ml of 10 mM $NaHCO_3$ [pH 8.5]). The reaction was started with $Na_2S_2O_4$ and stopped by vortexing to oxidize the MV^o (no zinc acetate was added).

Cyanide inhibition assays were run by modification of the procedure outlined in Table I and the above paragraph (through NO_2^- determination). Phosphate and KNO_3 were made as separate stock solutions and the reaction mixtures contained 270 μl 28 mM K^+-phosphate and 30 μl of either 170 mM (iNR, c_2NR) or 330 mM (c_1NR) KNO_3. Cyanide stock solutions were made in the absence of NaOH. Assays were started either directly with enzyme or, after a 5 min prereduction treatment of enzyme in the presence of NAD(P)H and KCN, with KNO_3. After 12 min the reaction was stopped with 50 μl zinc acetate.

Complementation Analysis. Complementation experiments were run as described by Narayanan *et al.* (21) with the following modifications. Mutant (nar2a) barley (*Hordeum vulgare* L.) seed were germinated and grown at 14 to 16°C and watered daily with 0.5X basic nutrient solution containing 15 mM $NaNO_3$ (12). Two days before harvest the NO_3^- concentration was increased to 17.5 mM. Seedlings were harvested at 7 d after planting. The purified soybean NR forms and commercial xanthine oxidase (Grade III, Sigma Chemical Co.[4]) were diluted to 0.25X and 30 μg/ml, respectively, with the phosphate buffer. After heat treatment, the MoCo sources (soybean NR forms and xanthine oxidase) were used, without centrifugation, for complementation.

Protein. Protein was determined by the method of Bradford (3) with BSA as a protein standard.

RESULTS

Each purified NR form (iNR, c_1NR, c_2NR) exhibited its expected preference for either NADH or NADPH as well as the partial activities ($FMNH_2$-NR, MV^o-NR, NADH-Cyt *c* reductase) normally associated with higher plant NR (Table II). The partial activities were consistently highest for c_1NR and iNR followed by c_2NR when compared with their respective maximum NAD(P)H-NR activities. Caution is advised in comparing partial activities of one NR form against that of another since (a) the assays were optimized, with saturating substrates and optimal pH, for NAD(P)H-NR activity and not for partial activities and (b) the three preparations used may have contained different amounts of partially inactivated enzyme.

Treatment with pHMB resulted in similar inhibition of the three NR forms and the effect could be prevented by increasing

[4] Mention of a trademark, vendor, or proprietory 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.

Table II. *NAD(P)H-NR and Partial Activities of Purified Soybean Leaf Nitrate Reductase Forms*

Data are means of two replicates ± SD with three parallel determinations. Cyt *c* reductase assays did not have parallel determinations.

NR Form	Nitrate Reductase				Cyt *c* Reductase	
	NADH	NADPH	$FMNH_2$	MV°	NADH	NADPH
	units·mg⁻¹ protein					
iNR	9.3 ± 0.1	0.7 ± 0.1	12.2 ± 0.0	12.8 ± 0.4	300 ± 24	24 ± 12
c_1NR	3.8 ± 0.4	5.8 ± 0.2	7.8 ± 1.6	7.3 ± 0.4	444 ± 24	468 ± 36
c_2NR	9.4 ± 0.2	2.4 ± 0.3	4.3 ± 1.4	4.6 ± 0.3	216 ± 12	96 ± 24

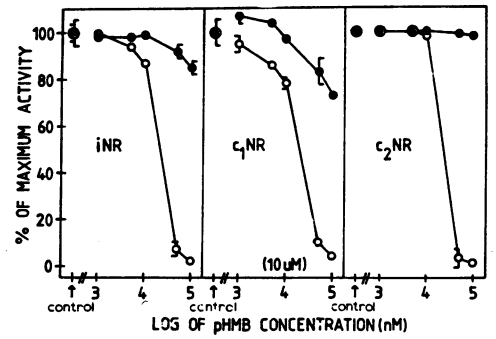

FIG. 1. Effect of various pHMB concentrations on NAD(P)H-NR activities of purified soybean leaf nitrate reductase forms. Enzymes were purified through hydroxylapatite (iNR) or blue sepharose (c_1NR, c_2NR). Relative to iNR, hydroxylapatite- and blue sepharose-purified c_1NR and c_2NR responded similarly to pHMB (data not shown) and saturation of c_1NR with KNO_3 did not significantly alter its response to pHMB (data not shown). The DTT assay mixture concentrations were 105 μM (●) and 10.5 μM (○). Specific activities (units/mg protein) for control assays in presence of 105 μM and 10.5 μM DTT, respectively, were 1.0 and 0.9 (iNR), 0.16 and 0.14 (c_1NR), and 0.28 and 0.28 (c_2NR). Data are means of two replicates ± SD. The SD was within the data point if not shown.

the concentration of DTT (Fig. 1). Crude extracts from leaves of urea-grown wild-type and NO_3^--grown nr_1 plants were inhibited by pHMB in a manner similar to that displayed by the purified enzymes (24). The major site of pHMB inhibition was associated with initial electron transfer through the purified enzymes (*e.g.* from NAD(P)H to Cyt *c*) since an almost complete inhibition of NAD(P)H-NR activity by pHMB resulted in no more than a 40% decrease in MV°-NR activity (Table III). Thus all forms, whether in purified or crude states, have sulfhydryl sites which react with pHMB leading to blockage of initial electron transfer through the enzymes.

Cyanide differentially inhibited the three NR forms with iNR being most susceptible followed by c_2NR and c_1NR, respectively (Fig. 2, filled symbols). Crude extracts of leaves from urea-grown wild-type and NO_3^--grown nr_1 plants mimicked c_2NR and iNR, respectively, in their response to CN^- (24). The site of CN^- inhibition was the terminal moiety of the purified enzymes since diaphorase (NADH-Cyt *c* reductase) activity was uninhibited in the presence of 76 μM KCN (24). Another inhibitor, HCO_3^-, which also affects the terminal site of electron transfer by competing with NO_3^- (23), also affected the purified cNR forms differently than the iNR form. The two cNR forms were completely inhibited by a 50 mM K_2CO_3 concentration while iNR was unaffected. These results, using two different inhibitors, support the concept that the terminal moieties of c_1NR, c_2NR, and iNR differ physically (structurally and/or conformationally) from one another.

To further investigate for an altered MoCo in the two cNR forms, all forms (iNR, c_1NR, and c_2NR) were prereduced with NAD(P)H in the presence of KCN and absence of NO_3^-. (Cyanide inhibits binding to the reduced form of the Mo in *Chlorella* NR [29, 30]). A 5 min prereduction treatment resulted in a greater decrease in activity for each NR form compared with the nonreduced controls (Fig. 2, open and closed symbols, respectively). This indicated the presence of a MoCo in each soybean NR form (prereduction affects all forms), but that the environment around the MoCo was different for each form (differential effect of CN^- without prereduction). The iNR response to CN^- was similar to that displayed by most NRs.

Support for the hypothesis that the MoCo was present but that the environment around it was different for each NR was obtained through complementation experiments between the purified soybean NR forms and the barley MoCo deficient mutant, nar2a (Table IV). All of the heat-treated purified NR forms, as well as commercial xanthine oxidase, released a factor which was able to complement the barley apoprotein. Among the NR forms, c_1NR had the greatest percent of units which complemented. Analysis, by starch gel electrophoresis, for contamination of the purified NR forms by other MoCo-containing enzymes such as xanthine oxidase and dehydrogenase, indicated a maximum contamination of 1.25% of the complementing activity displayed by commercial xanthine oxidase (24; Table IV). This amount of contamination cannot account for the high levels of complemen-

Table III. *Effect of 50 μM pHMB on NAD(P)H- and MV°-NR Activities of Purified Soybean Leaf Nitrate Reductase Forms*

Pyridine nucleotide electron donors were NADH, NADPH, and NADH for iNR, c_1NR, and c_2NR, respectively. The DTT concentration was 10 μM or less during assays. Enzymes were purified through hydroxylapatite. Specific activities (units/mg protein) for NAD(P)H- and MV°-NR activities, respectively, in the absence of pHMB, were 1.4 and 0.9 (iNR), 1.6 and 0.6 (c_1NR), and 2.2 and 0.6 (c_2NR). Data are means of two replicates ± SD.

NR Form	NAD(P)H-NR		+pHMB/−pHMB	MV°-NR		+pHMB/−pHMB
	−pHMB	+pHMB		−pHMB	+pHMB	
	units × 10^2·*ml⁻¹*		%	*units* × 10^2·*ml⁻¹*		%
iNR	1.70 ± 0.14	0.09 ± 0.01	5	1.16 ± 0.08	0.78 ± 0.09	67
c_1NR	1.79 ± 0.04	0.18 ± 0.01	10	0.66 ± 0.02	0.60 ± 0.07	91
c_2NR	2.28 ± 0.24	0.07 ± 0.03	3	0.63 ± 0.05	0.38 ± 0.06	60

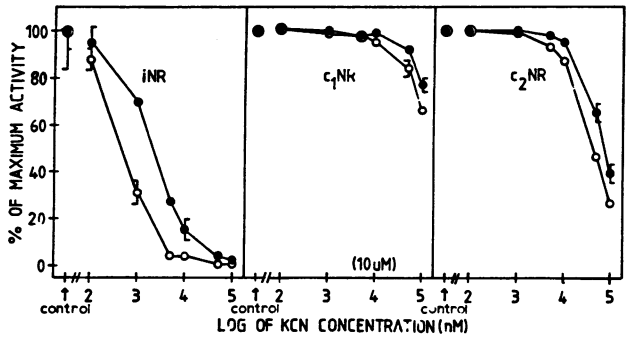

FIG. 2. Effect of various KCN concentrations on NAD(P)H-NR activities of purified soybean leaf nitrate reductase forms. Enzymes were purified through hydroxylapatite (iNR, c_1NR) or blue sepharose (c_2NR). Relative to iNR and c_1NR, hydroxylapatite- and blue sepharose-purified c_2NR showed no difference in response to KCN (data not shown), and saturation of c_1NR with KNO_3 did not significantly alter its response to KCN (data not shown). Assays were started either by addition of enzyme (●) or by addition of KNO_3 after 5 min preincubation of enzymes with NAD(P)H and KCN (○). Specific activities (units/mg protein) for control assays with and without preincubation, respectively, were 0.22 and 0.28 (iNR), 1.23 and 1.21 (c_1NR), and 0.33 and 0.33 (c_2NR). Data are means of three replicates ± SD. The SD was within the data point if not shown.

Table IV. *Complementation Analysis between MoCo Donors (Purified Soybean NR Forms and Commercial Xanthine Oxidase) and Apoprotein of the Barley MoCo Deficient Mutant, nar2a*

Nitrate reductase forms were purified through hydroxylapatite. Specific activities (units/mg protein) for soybean NR forms prior to complementation were 5.6, 0.8, and 1.0 for iNR, c_1NR, and C_2NR, respectively. The heat treatment involved rapid heating of the enzymes in phosphate buffer for the designated time in a 70°C water bath. The samples, in glass test tubes, were then rapidly cooled in ice water and used immediately as cofactor donors (21). Data are means of two replicates ± SD. Analysis of each soybean NR form was repeated at least once.

Molybdenum Cofactor Donor[a]	Nitrate Reductase Activity after Complementation Cofactor Pretreatment		Complementation[b]
	45 s heating	90 s heating	
	μmol NO_2^-·g^{-1} fresh wt·h^{-1}		%
None[c]	1.45 ± 0.13	1.58 ± 0.22	
Xanthine oxidase	4.78 ± 0.23	4.12 ± 0.11	
iNR	2.75 ± 0.29	2.66 ± 0.46	1
c_1NR	2.79 ± 0.49	3.15 ± 0.03	23
c_2NR	2.17 ± 0.40	2.35 ± 0.02	3

[a] Apoprotein donor was nar2a for all assays and all cofactor donors had no detectable activity after heat treatment. [b] Based on the units of activity produced after complementation of the nar2a apoprotein with the heat released (mean of 45 and 90 s heating period) MoCo from the purified soybean NR forms, divided by the units of purified soybean NR originally added, before heat treatment, to each complementation assay. Activities produced after complementation were corrected for endogenous activity associated with the nar2a apoprotein source. [c] Control treatment which consisted of heat treated phosphate buffer and no source of molybdenum cofactor other than residual MoCo associated with the nar2a apoprotein source.

tation displayed by the three NR forms thereby eliminating the xanthine oxidizing enzymes from any role during these experiments. Thus, from the CN^- inhibition and complementation studies it appears that the cNR forms, as well as iNR, contain cofactors with characteristics (heat stability, susceptibility to CN^-, and ability to complement with a barley NR defective in MoCo) similar to that of the MoCo found in NR from other higher plants. The differential effect of CN^- and differential abilities to complement the MoCo-deficient barley NR, however, indicate alterations in the terminal cofactor-containing portions of these enzymes.

DISCUSSION

The purified soybean NR forms (iNR, c_1NR, c_2NR) displayed pyridine nucleotide specificities (Table II) similar to those reported by Streit *et al.* (32). In addition, each form exhibited the partial activities characteristic of all eukaryotic nitrate reductases presently analyzed (Tables II, III; 9). Robin *et al.* (27) and Streit *et al.* (32) have reported immunological and kinetic data which indicate that c_1NR and the previously characterized NADPH:NR (5, 17) are identical. The lack of pyridine nucleotide specificity by the NAD(P)H-Cyt *c* reductase (diaphorase) activity of both c_1NR (Table II) and NADPH:NR (5) supports this hypothesis.

Mercurial compounds (pCMB, pHMB) inhibit the NAD(P)H-NR and diaphorase activities of higher plant NR while allowing MV°-NR activity to continue (7, 9, 15). Evans and Nason (8), who were the first to study this effect on higher plant NR, found that cysteine could prevent this inhibition. This indicated that free thiol groups were necessary for diaphorase activity. However, the absence of isozyme identification in their soybean extracts made it impossible to determine which NR form or forms they were characterizing. In our study, pHMB inhibited iNR, c_1NR, and c_2NR identically and the effect could be prevented by a reducing agent (DTT) in the classical manner (Fig. 1). In addition, although somewhat inhibited for iNR and c_2NR, MV°-NR activity continued in the absence of NAD(P)H-NR activity (Table III). Thus, each form has free thiol groups, necessary for diaphorase activity, with identical susceptibilities to pHMB.

Although one objective of this work was to characterize the three soybean NR forms in terms of partial activities and response to inhibitors, another major objective was to determine whether each form contained the normal molybdenum-containing MoCo. The MoCo has been considered to be a ubiquitous component of all assimilatory NRs (7, 9). The strong inhibition of purified c_1NR and c_2NR but not iNR by HCO_3^-, a compound shown to be a competitive inhibitor of NO_3^- for cNR activity in crude extracts (23), supports the possibility of altered terminal moieties in these two NR forms. An indication of the presence of a MoCo in the three NR forms comes from analysis of the MV°-NR activity data in the presence of 50 μM pHMB (Table III). Although MV°-NR activity continued with pHMB present, a considerable amount of inhibition (30–40%) occurred for iNR and c_2NR activity. The c_1NR activity was only slightly inhibited but later experiments, run in the presence of 100 μM pHMB, resulted in an approximately 35% inhibition for all forms (data not shown). Hewitt and Notton (15) noted that high concentrations of mercurials can inhibit MV°-NR activity and suggested that Mo-S bonds in the terminal portion of the enzyme may be the site of this inhibition. Cramer *et al.* (6), using x-ray absorption edge and extended x-ray absorption fine spectrum (EXAFS) spectroscopy, have shown initial evidence of Mo-S ligands for both assimilatory NR from *Chlorella* and dissimilatory NR from *E. coli*. They also determined that significant structural differences existed between the enzymes at the Mo site under certain conditions. It is interesting to speculate that the decreased MV°-NR activity of iNR, c_1NR and c_2NR, in the presence of high levels of pHMB, may be due to damage of Mo-S bonds in the terminal portion of these enzymes.

The CN^- inhibition experiments supplied stronger evidence for the presence of a MoCo in each NR form, with each Mo site having unique properties (Fig. 2; 24). The mode of CN^- inhibition in molybdoenzymes which do not possess a terminal sulfur ligand, such as sulfite oxidase and NR, involves binding of CN^-

to the reduced form of Mo with subsequent production of an over-reduced, nonactive Mo (15, 29, 30). In our studies, CN^- affected the terminal portion of the three NR forms (diaphorase activity was unaffected in the presence of CN^- [24]). A 5 min prereduction treatment with NAD(P)H in the presence of KCN resulted in greater inhibition of activity for each form, compared with the respective nonprereduced activity (Fig. 2, open *versus* closed symbols). Thus, iNR, c_1NR, and c_2NR all responded to CN^- in a manner consistent with the presence of a MoCo in each NR form. However, the differential inhibition of each NR form by CN^- (iNR > c_2NR > c_1NR; Fig. 2) supports the findings of Jolly *et al.* (17) and indicates that the environment around the Mo is different for each form. The high sensitivity of iNR to low concentrations of CN^- is similar to that observed for NR from other higher plants (1, 20, 26) and further supports the contention (23, 32) that this form is similar in all characteristics to the highly studied assimilatory NADH:NR (EC 1.6.6.1) found in all presently studied higher plants (9).

Complementation analysis showed that each heat-treated soybean NR fraction, as well as xanthine oxidase, released heat-stable cofactors which complemented the barley (nar2a) apoprotein (Table IV). Since the nar2a apoprotein contains a functional flavin (measured by diaphorase activity [31]), and a functional heme (xanthine oxidase, which complements, contains no heme [4]), the complementing soybean NR forms must be supplying cofactors with structures similar to a normal MoCo. The possibility of contamination of the purified NR fractions by other MoCo-containing enzymes does exist but is minimal (24). A contamination problem not so readily discounted involves contamination of c_2NR by c_1NR. On a unit basis, c_1NR complements approximately 7 times better than c_2NR and therefore a 10% contamination could account for a major portion of the complementation exhibited by the c_2NR fraction. Although we cannot discount this possibility, the more similar sensitivities of the terminal moieties of c_2NR and iNR to CN^- indicates that c_2NR may have a cofactor-protein configuration more closely related to iNR; the latter having only a 1% complementation rate. The high ability of c_1NR to complement, on a per cent unit basis, compared with the other NR forms indicates that c_1NR has either a slower reaction rate or releases its cofactor more readily during heat treatment. Since the relative purities of the NR forms are unknown, it is impossible to distinguish between these possibilities.

Although the three soybean NR forms (iNR, c_1NR, and c_2NR) are similar in many respects to most assimilatory NR enzymes, the dissimilarities noted indicated that structural or conformational differences do exist in the terminal cofactor-containing portion of each enzyme. The presence of Mo in the terminal cofactors of each form seems apparent, based on CN^- inhibition and complementation analyses, but this aspect remains to be proven by more direct methods.

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LITERATURE CITED

1. ARYAN AP, RG BATT, W WALLACE 1983 Reversible inactivation of nitrate reductase by NADH and the occurrence of partially inactive enzyme in the wheat leaf. Plant Physiol 71:582–587
2. ASLAM M 1982 Differential effect of tungsten on the development of endogenous and nitrate-induced nitrate reductase activities in soybean leaves. Plant Physiol 70: 35–38
3. 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
4. BRAY RC 1980 The reactions and the structures of molybdenum centers in enzymes. *In* A Meister, ed, Advances in Enzymology and Related Areas of Molecular Biology, Vol 51. John Wiley & Sons, New York, pp 107–165
5. CAMPBELL WH 1976 Separation of soybean leaf nitrate reductases by affinity chromatography. Plant Sci Lett 7: 239–247
6. CRAMER SP, LP SOLOMONSON, MWW ADAMS, LE MORTENSON 1984 Molybdenum sites of *Escherichia coli* and *Chlorella vulgaris* nitrate reductase: a comparison by EXAFS. J Am Chem Soc 106: 1467–1471
7. DUNN-COLEMAN NS, J SMARRELLI, JR, RH GARRETT 1984 Nitrate assimilation in eukaryotic cells. Int Rev Cytol 92: 1–50
8. EVANS HJ, A NASON 1953 Pyridine nucleotide-nitrate reductase from extracts of higher plants. Plant Physiol 28:233–254
9. GUERRERO MG, JM VEGA, M LOSADA 1981 The assimilatory nitrate-reducing system and its regulation. Annu Rev Plant Physiol 32: 169–204
10. HAGEMAN RH, DP HUCKLESBY 1971 Nitrate reductase from higher plants. Methods Enzymol 23: 491–503
11. HARPER JE 1974 Soil and symbiotic nitrogen requirements for optimum soybean production. Crop Sci 14: 255–260
12. HARPER JE 1981 Effect of chlorate, nitrogen source, and light on chlorate toxicity and nitrate reductase activity in soybean leaves. Physiol Plant 53: 505–510
13. HARPER JE, JC NICHOLAS 1978 Nitrogen metabolism of soybeans. I. Effect of tungstate on nitrate utilization, nodulation, and growth. Plant Physiol 62: 662–664
14. 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
15. 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
16. JOHNSON JL, BE HAINLINE, KV RAJAGOPALAN, BH ARISON 1984 The pterin component of the molybdenum cofactor: Structural characterization of two fluorescent derivatives. J Biol Chem 259: 5414–5422
17. JOLLY SO, WH CAMPBELL, NE TOLBERT 1976 NADPH- and NADH-nitrate reductases from soybean leaves. Arch. Biochem. Biophys 174: 431–439
18. KAKEFUDA G, SH DUKE, SO DUKE 1983 Differential light induction of nitrate reductases in greening and photobleached soybean seedlings. Plant Physiol 73: 56–60
19. LAHAV E, JE HARPER, RH HAGEMAN 1976 Improved soybean growth in urea with pH buffered by a carboxy resin. Crop Sci 16: 325–328
20. LEONG CC, TC SHEN 1979 Reversible inactivation of the nitrate reductase of rice plants. Experientia 35: 584–585
21. NARAYANAN KR, AJ MÜLLER, A KLEINHOFS, RL WARNER 1984 *In vitro* reconstitution of NADH: nitrate reductase in nitrate reductase-deficient mutants of barley. Mol Gen Genet 197: 358–362
22. NELSON RS, SA RYAN, JE HARPER 1983 Soybean mutants lacking constitutive nitrate reductase activity. I. Selection and initial plant characterization. Plant Phsyiol 72: 503–509
23. NELSON RS, L STREIT, JE HARPER 1984 Biochemical characterization of nitrate and nitrite reduction in the wild-type and a nitrate reductase mutant of soybean. Physiol Plant 61: 384–390
24. NELSON RS 1985 Biochemical characterizations of a soybean (*Glycine max* [L.] Merr.) nitrate reductase mutant and crude and purified nitrate reductases. PhD thesis. University of Illinois, Urbana-Champaign
25. NICHOLAS DJD, A NASON 1955 Role of molybdenum as a constituent of nitrate reductase from soybean leaves. Plant Physiol 30: 135–138
26. RELIMPIO AMA, PJ APARICIO, A PANEQUE, M LOSADA 1971 Specific protection against inhibitors of the NADH-nitrate reductase complex from spinach. FEBS Lett 17: 226–230
27. ROBIN P, L STREIT, WH CAMPBELL, JE HARPER 1985 Immunochemical characterization of nitrate reductase forms from wild-type (cv. Williams) and nr_1 mutant soybean. Plant Physiol 77: 232–236
28. 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
29. SOLOMONSON LP 1981 Cyanide as a metabolic inhibitor. *In* B Vennesland, EE Conn, CJ Knowles, J Westley, eds, Cyanide in Biology. Academic Press, New York, pp 11–28
30. SOLOMONSON LP, MJ BARBER, WD HOWARD, JL JOHNSON, KV RAJAGOPALAN 1984 Electron paramagnetic resonance studies on the molybdenum center of assimilatory NADH-nitrate reductase from *Chlorella vulgaris*. J BiolChem 259: 849–853
31. SOMERS DA, TM KUO, A KLEINHOFS, RL WARNER 1983 Nitrate reductase-deficient mutants in barley. Plant Physiol 71: 145–149
32. 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
33. WRAY JL, P FILNER 1970 Structural and functional relationships of enzyme activities induced by nitrate in barley. Biochem J 119: 715–725