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Some Characteristics of Nitrate Reductase from Higher Plants[1]

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Abstract. With respect to cofactor requirements, NADH, and $FMNH_2$ were equally effective as electron donors for nitrate reductase obtained from leaves of maize, marrow, and spinach, when the cofactors were supplied in optimal concentrations. The concentration of $FMNH_2$ required to obtain half-maximal activity was from 40- to 100-fold higher than for NADH. For maximal activity with the corn enzyme, 0.8 millimolar $FMNH_2$ was required. In contrast, NADPH was functional only when supplied with NADP:reductase and exogenous FMN (enzymatic generation of $FMNH_2$).

All attempts to separate the $NADH_2$- and $FMNH_2$-dependent nitrate reductase activities were unsuccessful and regardless of cofactor used equal activities were obtained, if cofactor concentration was optimal. Unity of NADH to $FMNH_2$ activities were obtained during: A) purification procedures (4 step, 30-fold); B) induction of nitrate reductase in corn seedlings with nitrate; and C) inactivation of nitrate reductase in intact or excised corn seedlings. The NADH- and $FMNH_2$-dependent activities were not additive.

A half-life for nitrate reductase of approximately 4 hours was estimated from the inactivation studies with excised corn seedlings. Similar half-life values were obtained when seedlings were incubated at 35° in a medium containing nitrate and cycloheximide (to inhibit protein synthesis), or when both nitrate and cycloheximide were omitted.

In those instances where NADH activity but not $FMNH_2$ activity was lost due to treatment (temperature, removal of sulfhydryl agents, addition of *p*-chloromercuribenzoate), the loss could be explained by inactivation of the sulfhydryl group(s) required for NADH activity. This was verified by reactivation with exogenous cysteine.

Based on these current findings, and previous work, it is concluded that nitrate reductase is a single moiety with the ability to utilize either NADH or $FMNH_2$ as cofactor. However the high concentration of $FMNH_2$ required for optimal activity suggests that *in vivo* NADH is the electron donor and that nitrate reductase in higher plants should be designated NADH:nitrate reductase (E.C. 1.6.6.1).

It is commonly reported (4, 5, 6, 17, 19, 23) that light is coupled to nitrate reduction via NADPH and that the enzyme is NAD(P)H:nitrate oxidoreductase (E.C. 1.6.6.2). In 1954, Nicholas and Nason (21) demonstrated that enzymatically reduced flavins would also serve as electron donors for the NADPH:nitrate oxidoreductase (E.C. 1.6.6.3) obtained from *Neurospora*. Although this enzyme catalyzed 3 electron transfer steps, namely NADPH →FAD→molybdenum→nitrate, evidence suggested that it was a single protein, a belief still held 10 years later (10).

With respect to higher plants, Stoy (26) reported that riboflavin, photoreduced under anaerobic conditions, was more effective as an electron donor to nitrate reductase than was NADH. In 1965, Paneque *et al.* (22) reported that free $FMNH_2$ and $FADH_2$ are the natural cofactors for nitrate reduction in higher plants and that NAD(P)H:nitrate oxidoreductase is a mixture of 2 enzymes—NADP:reductase and nitrate reductase which they classified as $FMNH_2$ ($FADH_2$):nitrate oxidoreductase (a molybdoprotein). In a companion paper (15) it was stated that the enzymic machinery needed for the reduction of NO_3^- to NH_4^+ is contained in the chloroplasts. These 2 reports have been consolidated and proposed (16) as a logical explanation for the commonly observed light enhanced reduction of nitrate.

In contrast to these reports that link light to nitrate reduction via photoreduced electron donors, Kessler's work with algae (13) suggests that nitrate reduction is more dependent on the production of photosynthate while nitrite reduction is more directly related to the photochemical process. In a subsequent review (14) Kessler suggests that photosynthetically generated NADPH would not serve as the electron donor for nitrate reductase from *Ankistro-*

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desmus and that the lack of photosynthate to produce NADH was responsible for the inability of the algae to reduce nitrate. The work of Beevers *et al.* (2) shows that nitrate reductase from 16 species of higher plants has a specific or preferential requirement for NADH rather than NADPH as cofactor. These findings and other work (24) that demonstrates that nitrate reductase is not localized within the chloroplasts support Kessler's view (13) that nitrate reduction is not coupled directly to light.

Recently Paneque and Losada (23) confirmed the earlier report of Beevers *et al.* (2) that NADH, but not NADPH, could serve directly as electron donor for a highly purified nitrate reductase from spinach. They had to add NADP:ferredoxin reductase and free FMN to the system before NADPH could donate electrons for nitrate reduction, while no additions were required when NADH was the electron donor. However, they suggest that NADH is coupled to the $FMNH_2(FADH_2)$:nitrate oxidoreductase via NAD:reductase and an unidentified cofactor.

The objectives of this study were to determine whether nitrate reduction in higher plants is achieved by: A) 2 separate nitrate reductases which differ in cofactor requirement, B) a single enzyme (possibly composed of tightly coupled subunits), that can directly utilize both NADH or reduced flavins as electron donors, or C) FMN(FAD-)H_2:nitrate oxidoreductase which is capable of utilizing reduced pyridine nucleotides only indirectly via separate pyridine nucleotide reductases and flavins or an unidentified coupling factor.

Experimental Procedure

Plant Culture. Corn (*Zea mays* L. variety Hy2 × Oh7) and marrow (*Cucurbita pepo* L. variety Early White Bush) used for induction studies were planted and grown as previously described (3) with the following exceptions: Vermiculite (Zonolite Company, Chicago, Illinois) was used as the supporting medium. The nutrient solution used for daily irrigation had the following composition, in mmoles per liter: K_2SO_4, 0.25; KH_2PO_4, 1.0; $MgSO_4$, 4.0; $CaCO_3$, 2.0; $(NH_4)_2CO_3$, 5.0; Fe^{+3} (as Chel-138, Geigy Agricultural Chemical Company, Yonkers, New York) 0.3; in μmoles per liter: H_3BO_3, 23; $MnCl_2$, 46; $ZnSO_4$, 15; $CuSO_4$, 1.6; H_2MoO_3, 0.7. The pH was adjusted to 7.5 with HCl. The addition of ammonium ions to the nutrient solution did not appreciably increase the endogenous (initial level) of nitrate reductase in the shoots and cotyledons. The growth chamber environment was: 40,000 lux, 15 hour day at 28° and 9 hour night at 24°.

Plant material, including spinach (*Spinacia oleracea* L.), with a high level of nitrate reductase, used for characterization studies, was grown as described in the preceding paragraph, except the nutrient solution was supplemented with 0.05 M KNO_3 or grown in soil in the greenhouse as previously described (2).

Induction of Excised Seedlings and Cotyledons. Induction was accomplished as previously described (3) except that 20 mM potassium phosphate buffer, pH 4.0, was always used in the induction medium.

Extraction and Assay of Nitrate and Nitrite Reductases. The extraction of the enzymes was as previously described (3) for corn. Similar extraction procedures were used for other material with minor modifications; *e.g.*, the pH was adjusted to 8.0 and the cysteine concentration lowered to 1.0 mM for spinach and marrow.

When NADH was used as the electron donor, nitrate reductase was assayed by a modification (3) of the original method of Evans and Nason (6) except that the phosphate buffer was 25 mM. The reaction was terminated and interfering substances eliminated by adding 0.1 ml of 1.0 M zinc acetate and 3.0 ml 95% ethanol (20) prior to assay for nitrite.

When $FMNH_2$ was used as the electron donor, nitrate reductase was assayed by a modification of the procedure of Paneque *et al.* (22). The basic assay mixture contained in μmoles in a 2 ml volume (final), KNO_3 — 20, FMN — 1.6, potassium phosphate — 50 (pH 7.5), and an appropriate amount of enzyme. The reaction was initiated either by adding sodium hydrosulfite ($Na_2S_2O_4$) (8.0 μmoles in 0.3 ml of 20 mM phosphate buffer, pH 7.5) or by adding $FMNH_2$ prereduced in the dark with hydrogen and palladized asbestos to the evacuated tubes. With enzymatically reduced FMN, the reaction was initiated by adding NADPH (2.0 μmoles) and NADP:reductase (0.1 ml; 0.2 mg protein). Initially, all the reaction tubes were stoppered and evacuated; however, this precaution was shown to be unnecessary when sodium hydrosulfite was employed. After 15 minutes at 28°, the reaction was terminated by shaking vigorously for 20 seconds on a Vortex mixer. Zinc acetate and ethanol were then added and the nitrite determined as previously described.

Nitrite reductase was assayed by the procedure described by Joy and Hageman (12) except that the concentration of dithionite and benzyl viologen was doubled.

Purification of Nitrate Reductase. Nitrate reductase was extracted as previously described except that only 2 ml of extraction medium was added for each gram of fresh weight tissue. The enzyme was extracted from 8-day old marrow cotyledons, 10-day old corn leaves, or 30-day old spinach leaves. The procedure used for purification of nitrate reductase from marrow is as follows: The homogenate was strained through 4 layers of cheesecloth and centrifuged (20 min, 30,000 × *g*). The desired amount of solid $(NH_4)_2SO_4$ was added (slowly and with pH maintained at pH 7.5 by addition of KOH, 0.05 N), to the supernatant to obtain the various fractions. The precipitate was allowed to form for

15 minutes and then collected by centrifugation (15 min, 15,000 × *g*). The precipitates were in all cases dissolved in 25 mM phosphate (SP) buffer, (pH 7.5 and 1.0 mM with respect to cysteine) and debris removed by centrifugation. The 25 to 42% $(NH_4)_2SO_4$ precipitate was dissolved in a minimal amount of SP buffer and mixed with an equal volume of calcium phosphate gel (pH 7.5, 180 mg dry wt per ml). The mixture was stirred occasionally for 10 minutes and centrifuged (5 min, 1000 × *g*). The precipitate was washed with SP buffer (volume one-half that of the discarded supernatant), and recollected by centrifugation (5 min, 1000 × *g*). The enzyme was then eluted with 0.1 M sodium pyrophosphate, pH 7.5. Ammonium sulfate fractionation was repeated as above and the desired precipitate dissolved in a minimal volume of SP buffer. This solution, after centrifugal clarification, was applied to calcium phosphate gel-columns (1.5 cm wide and 1.5 cm long) and each column washed with phosphate buffers (7.5 ml, 25 mM and 6.0 ml, 50 mM) and eluted with pyrophosphate (6.5 ml, 0.1 M). The wash and elution buffers were at pH 7.5 and 1.0 mM with respect to cysteine for both marrow and spinach. Although the sequence of steps was different, the same general procedure was used with the enzyme from corn and spinach leaves. In some experiments the crude extract was passed through a DEAE cellulose column to remove ferredoxin. In these instances the columns were equilibrated with the extraction medium (adjusted to pH 7.5). With the corn enzyme, the calcium phosphate column was washed with 50 mM phosphate, pH 7.5 and 5 mM with respect to cysteine and eluted with 0.1 M phosphate, pH 7.5 and 10 mM with respect to cysteine. All operations were performed at 0 to 3°. The amounts of ammonium sulfate used for fractionation are expressed as a percentage of saturation.

Preparation and Assay of NADP: and NAD: reductases. The NADP:reductase was prepared from greenhouse-grown corn and spinach leaf tissue by the following procedure: 150 grams of leaves were homogenized in 300 ml of 25 mM phosphate buffer (pH 8.8 for corn and pH 8.0 for spinach) containing 5 mM EDTA. The homogenate was strained through cheesecloth and centrifuged 20 minutes at 30,000 × *g*. The supernatant was heated to 60° for 5 minutes, strained through cheesecloth, and centrifuged at 30,000 × *g* for 20 minutes. The supernatant was taken to 65% ammonium sulfate and centrifuged 10 minutes at 10.000 × *g*; the precipitate was suspended in 6 ml of 25 mM phosphate, pH 7.5. The suspension was centrifuged 10 minutes at 600 × *g* and the precipitate was discarded. The NADP:reductase was frozen in small batches at −20°. These preparations also contained NAD:reductase but not nitrate reductase activity.

NAD: and NADP:reductase activities were assayed spectrophotometrically using dichlorophenolindo phenol dye (DCIP) as the electron acceptor. The cuvette contained in μmoles: phosphate buffer, pH 8.0, 1000; DCIP, 0.15; NADH or NADPH, 0.67; an appropriate amount of enzyme; and H_2O to final volume of 3 ml. The reduction of the dye was followed at 620 mμ with a recording Beckman DB spectrophotometer for 2 minutes. Appropriate corrections were made for non-enzymatic reduction of the dye by NADH or NADPH.

Protein content of the extracts was determined by digesting the 5% trichloroacetic acid precipitable material followed by nesslerization (8).

Table I. *Comparison of NADH and $FMNH_2$ as Electron Donors for Nitrate Reductase*

Nitrate reductase and NADP:reductase were purified according to the procedures described in Methods. The complete mixture for the NADH and enzymatically reduced FMN assay systems contained: in μmoles per 2 ml final volume; phosphate buffer, pH 7.5, 50; nitrate, 20; FMN, 0.4; and either NADH, 0.4 or NADPH, 2.0; and in mg protein per assay; nitrate reductase, 0.03 and 0.05, for corn and spinach, respectively; and NADP:reductase, 0.6. When FMN was reduced chemically, the complete system contained in μmoles: phosphate buffer, pH 7.5, 50; nitrate, 20; FMN, 1.6; $Na_2S_2O_4$, 8.0; and nitrate reductase as described above. All assays were run anaerobically in this experiment.

Assay deletions	Nitrate reductase activity					
	Corn			Spinach		
	NADH	$FMNH_2$		NADH	$FMNH_2$	
		Chemical[1]	Enzymatic[2]		Chemical[1]	Enzymatic[2]
	mμmoles NO_2^-, mg protein^{-1}, min^{-1}					
None	255	245	239	127	130	120
Nitrate reductase	0	0	0	0	0	0
NADP:reductase	290	...	11	116	...	13
FMN	236	...	28	97	...	45
NADP:reductase and FMN	249	...	8	...	...	...

[1] FMN reduced chemically by dithionite ($Na_2S_2O_4$).
[2] FMN reduced enzymatically by NADPH and NADP:reductase.

Results and Discussion

Comparison of NADH, NADPH, and $FMNH_2$ as Electron Donors for Nitrate Reductase. The data (table I) show that NADH and $FMNH_2$ were equally effective electron donors to purified nitrate reductase from corn and spinach leaves. Addition of exogenous enzymes or flavins was not required with NADH, but NADPH was not effective as an electron donor unless coupled to nitrate reductase via NADP:reductase and free FMN. Since NADPH was not effective alone, its function was to reduce FMN because chemically and enzymatically reduced FMN were equally effective in donating electrons to the system. Although the NADH reaction proceeds equally well aerobically, anaerobic conditions were employed for both NADPH and NADH assays in this experiment to prevent air oxidation of the enzymatically reduced FMN in the NADPH assay.

The activity noted in spinach when FMN was omitted from the complete system (45 units) suggested that NADP:reductase was coupling directly to the nitrate reductase. Since repeated attempts to verify this finding were unsuccessful and Losada *et al.* (15) also found no direct coupling, it was concluded that this result was an artifact presumably due to endogenous FMN in this sample.

Purification of Nitrate Reductase. No separation of NADH and $FMNH_2$:nitrate reductase was obtained by purification procedures that achieved 16- and 29-fold enrichment of the enzyme from marrow cotyledons and corn leaves, respectively (table II). The activities per unit of protein obtained with both electron donors are comparable to those reported by Paneque *et al.* (22). These results would imply that nitrate reductase is a single protein capable of utilizing NADH or $FMNH_2$ as electron donors, unless it is conceived that 2 enzymes are present, and are so nearly identical in physical properties that separation cannot be achieved by common purification procedures.

Substrate Induction. Since nitrate reductase can be induced in excised young corn seedlings by submerging them in a medium containing nitrate (3), this technique was utilized to show that $FMNH_2$ and NADH activities were induced at equal rates over a 7.5 hour period (fig 1). These experiments suggest, but do not prove the enzyme to be a single protein capable of dual cofactor utilization, only because of the possibility of simultaneous induction. The earlier report (25) of unequal and slower induction of $FMNH_2$:nitrate reductase activity in corn seedlings was incorrect. This result was obtained because greater amounts of $FMNH_2$ are required

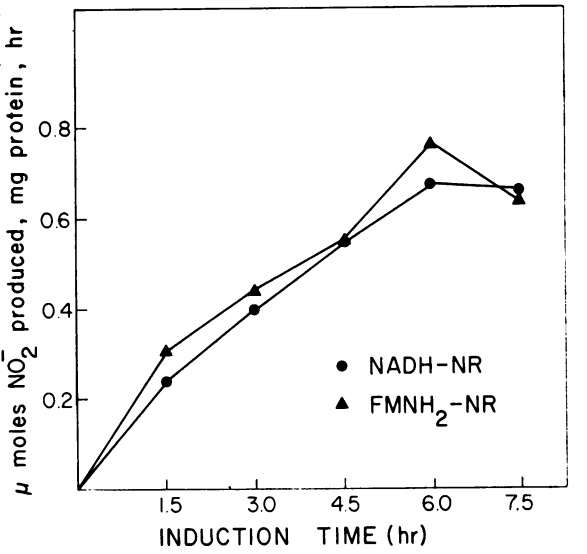

FIG. 1. Comparison of rate of induction of $FMNH_2$: and NADH:nitrate reductase activities in excised 9-day old nitrate deficient corn seedlings. Induction and enzyme assays were as described in methods. Rate of induction was determined by removing 3 samples from the induction media at 1.5 hour intervals and assaying for NADH: and $FMNH_2$:nitrate reductase activities.

Table II. *Purification of Nitrate Reductase*

Nitrate reductase from marrow cotyledons and corn leaves was purified as described in Methods. NADH: and $FMNH_2$:nitrate reductase were assayed after each purification step.

Fraction	Volume	Amount of protein	Nitrate reductase activity $FMNH_2$	NADH
	ml	*mg/ml*	*mμmoles NO_2^-, min^{-1}, mg $protein^{-1}$*	
		Marrow		
Extract	450	3.3	27	23
$(NH_4)_2SO_4$ (25–42 % ppt.)	150	2.6	66	69
$CaPO_4$ gel-batch	103	0.8	118	149
$(NH_4)_2SO_4$ (25–42 % ppt.)	25	1.4	197	228
$CaPO_4$ gel-column	80	0.2	370	400
		Corn		
Extract	40	3.20	20	23
$CaPO_4$ gel-batch	11	0.51	132	154
$(NH_4)_2SO_4$ (0–50 % ppt.)	7	0.26	332	328
Sephadex	14	0.11	325	318
$CaPO_4$ gel-column	15	0.04	713	725

for optimum activity of nitrate reductase from corn than from spinach leaves (22).

Decay or Inactivation of Nitrate and Nitrite Reductases in Excised Corn Seedlings. Previous work has shown that synthesis of nitrate reductase is dependent upon the presence of nitrate (3), and cycloheximide can effectively inhibit nitrate reductase synthesis even in the presence of nitrate (11). It has also been noted that nitrate reductase from corn leaf tissue is short lived both *in vitro* (2) and *in vivo* (3, 7). These experimental procedures provided an approach in determining whether $FMNH_2$- or NADH-dependent nitrate reductase activity exhibited differential lability in excised corn seedlings.

The data presented in figure 2A show the loss of nitrate reductase from excised corn seedlings that were floated in standard induction medium containing cycloheximide. Similar decay rates were observed regardless of co-factor used for assay, thus permitting an estimate of a 4.2 hour half-life for nitrate reductase. When comparable seedlings were floated in the standard induction medium (cycloheximide omitted), little or no loss of nitrate reductase was observed. Again, similar results were obtained regardless of co-factors.

Similar results were obtained in a companion experiment where excised seedlings that contained a minimal level of nitrate were floated in phosphate buffer (20 mM, pH 4.0) without nitrate or cycloheximide (fig 2B). Similar decay rates were obtained with NADH or $FMNH_2$ assay and the half-life was 3.5 hours. This demonstrated that cycloheximide did not cause breakdown of nitrate reductase.

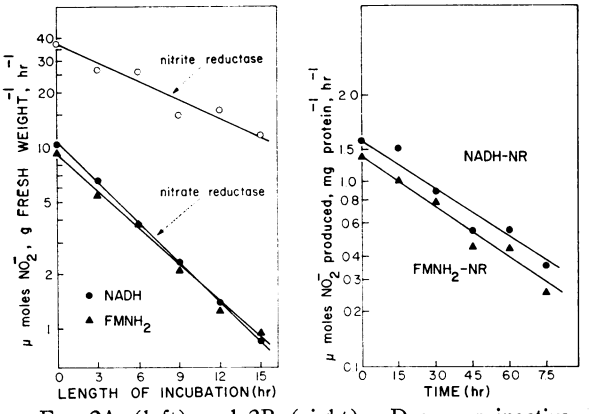

FIG. 2A (left) and 2B (right). Decay or inactivation rates of nitrate and nitrite reductases obtained with excised corn seedlings. Data in 2A show loss of activity from seedlings preinduced and containing high levels of nitrate and nitrite reductase activities prior to excision and placing in media containing nitrate and cycloheximide (50 μg/ml). Three samples were removed every 3 hours and assayed for nitrate and nitrite reductase activities (see methods). Data in 2B show loss of nitrate reductase activity from seedlings preinduced and containing high levels of nitrate reductase activity but minimal levels of nitrate prior to excision and placing in phosphate buffer (20 mM, pH 4) but without nitrate or cycloheximide. Three samples were removed at 1.5 hour intervals and assayed. All seedlings in these experiments were incubated at 35°, because this has been shown (3) to be the optimum temperature for induction.

In this and other experiments the higher (4-fold) initial level and slower loss of activity (9 hr half-life, fig 2A) of the nitrite reductase from the same extracts provide convincing evidence that nitrate reductase is the rate limiting step in the complete reduction of nitrate. Furthermore, nitrate can and does accumulate to high concentrations (5000 μg NO_3^-, g fr wt^{-1}) in corn leaf tissue with no apparent injury to the plant, whereas nitrite does not accumulate (not higher than 1 μg NO_2^-, g fr wt^{-1}). The apparent Km's for nitrate and nitrite are 1.4×10^{-4} M and 10^{-6} M, respectively.

Heat Induced Loss of Nitrate Reductase in Intact Seedlings. Mattas and Pauli (18) found that intact corn plants exposed to high temperature and moisture stress rapidly lost NADH-dependent nitrate reductase activity, concurrent with nitrate accumulation and cessation of protein synthesis. This work suggested that temperature might inactivate the sulfhydryl containing NAD:reductase without denaturing the $FMNH_2$($FADH_2$):nitrate reductase (23). If this selective denaturation could be demonstrated, evidence would be provided that the $FMNH_2$:nitrate reductase was not operative *in vivo*. Accordingly, corn seedlings were grown on a nitrate medium at normal temperatures (day 28°, night 24°), for 14 days, evaluated for nitrate reductase level, and half of the material was transferred to a comparable growth chamber, except for an increase in temperature (day 38°, night 34°). No differential loss of $FMNH_2$- or NADH-dependent nitrate reductase activity was noted after a 24 hour stress period although 60 % of the original activity was lost. In contrast, the seedlings maintained at the original temperature exhibited a 20 % increase in nitrate reductase activity. Thus heat induced loss of nitrate reductase *in vivo* did not differentially affect the efficiency of utilization of the 2 cofactors. This result again suggests a single enzyme capable of dual cofactor utilization.

Optimum pH of Assay. A pH of 7.5 was found to be optimum for maximal nitrate reductase activity with either $FMNH_2$ or NADH as electron donor.

Apparent Km Values. Apparent Km values for $FMNH_2$, NADH, and nitrate were determined (fig 3) with partially purified nitrate reductase preparations obtained from corn and spinach leaves. Higher concentrations of $FMNH_2$ (40- and 100-fold with the enzymes from spinach and corn, respectively) were required to achieve half-maximal rates of nitrate reduction than when NADH was used. When $FMNH_2$ was used as the electron donor, nitrate concentration required for half-maximal rates was greater (3- to 4-fold) than when NADH was used. Since dithionite, a strong reducing agent, was added directly to the $FMNH_2$:nitrate reductase

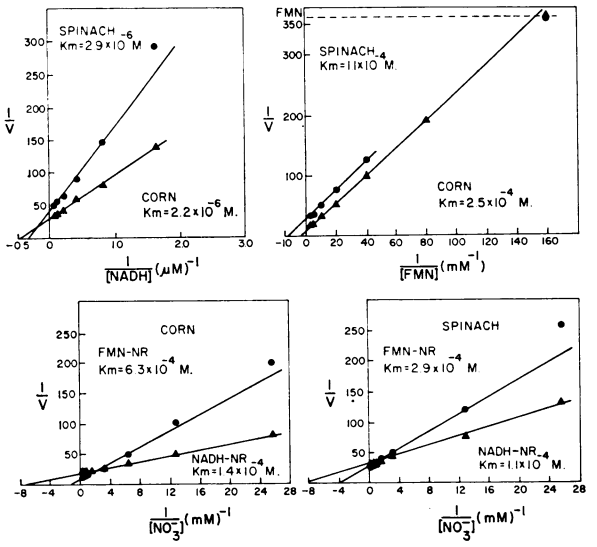

FIG. 3. Apparent Km values for NADH, $FMNH_2$, and NO_3^- obtained with partially purified nitrate reductases obtained from spinach and corn (see Methods). V_{max} for corn and spinach nitrate reductases were 144 and 155 mμmoles NO_2^-, mg protein^{-1}, min^{-1}, respectively. The amounts of enzyme used per assay were 0.04 and 0.03 mg for corn and spinach, respectively.

assay mixture, it could interact directly with the enzyme and thus alter the Km value. However, when FMN reduced by hydrogen gas and palladized asbestos prior to addition to evacuated (anaerobic) tubes was substituted for the dithionite-FMN mixture, similar Km values for nitrate were still obtained.

If free FMN is to participate *in vivo* as proposed (16) and implied (23), as the coupling factor to $FMNH_2$ ($FADH_2$) :nitrate reductase, these data suggest that maximum activity for corn would require 0.8 mM levels of reduced flavin.

Use of $FMNH_2$ and NADH Alone and Together for Nitrate Reduction. A purified preparation of nitrate reductase from corn was used to determine whether $FMNH_2$ or NADH is used preferentially by nitrate reductase when both are present in the reaction mixture. FMN was prereduced with hydrogen and palladized asbestos because dithionite and NADH together interfered with nitrate reduction. The rationale of the experiment was to equate the amount of NO_2^- produced (measured chemically) with the amounts of $FMNH_2$ and NADH oxidized (determined by absorbancy changes at 450 and 340 mμ, respectively) when cofactors were added separately and together. The results of such an experiment are presented in table III.

The addition of $FMNH_2$ and NADH together did not result in any significant increase in nitrate reduction over that obtained when the cofactors were added separately (table III). This implies that the same enzyme was accepting electrons from both NADH and $FMNH_2$. It is significant that no reduction of FMN was effected by NADH and nitrate reductase in the system when nitrate was either present or absent. Thus NADH donated its electrons directly to nitrate reductase and not indirectly via FMN. When both cofactors were present at optimal concentrations (130 μM for NADH and 800 μM for $FMNH_2$) the absorbancy changes indicated the oxidation of 129 and 112 mμmoles of NADH and $FMNH_2$, respectively, which combined provide the stoichiometrically required reducing capacity for the production of 241 mμmoles of nitrite that were formed. It should be noted that 2400 mμmoles of $FMNH_2$ were required for maximal activity whereas only 390 mμmoles of NADH were added.

Since it seems unlikely that reduced flavin concentrations are nearly millimolar *in vivo,* these data support the Km data in providing evidence that NADH is a more important electron donor than $FMNH_2$ for nitrate reductase in higher plants under physiological conditions.

Sulfhydryl Requirements. The initial work of Evans and Nason (6) established a sulfhydryl requirement for nitrate reductase in higher plants and in their review Hewitt and Nicholas (10) have depicted an active sulfhydryl site that could possibly be associated with bonding and function of pyridine nucleotide. This −SH group is sensitive to inhibition by *p*-chloromercuribenzoate (CMB). Avron

Table III. *Study of Cofactor Preference for Nitrate Reductase*

Cofactor (NADH and $FMNH_2$) oxidation by a purified nitrate reductase (0.03 mg protein per cuvette) from corn was followed spectrophotometrically in a Zeiss PMQ II spectrophotometer to determine if 1 of the cofactors is used preferentially when both are present. Absorbancy changes were obtained at minute intervals for 15 minutes. An aliquot was taken from each cuvette at the end of the 15 minute period for nitrite determination. Optimal concentrations (130 μM for NADH and 800 μM for $FMNH_2$) of cofactors were added to all reaction mixes (3 ml final volume). Because the oxidation of $FMNH_2$ also alters absorbancy at 340 mμ [Hemmerich *et al.* (9)] it was necessary to monitor all combinations [with and without nitrate, enzyme and cofactor (NADH and $FMNH_2$ alone and combined)] at both 340 and 450 mμ. Anaerobic conditions were used for all assays in this experiment. All assays were run in duplicate.

Cofactor(s) added to cuvette	mμmoles cofactor oxidized[1]		mμmoles nitrite produced[2]
	340 mμ	450 mμ	
NADH	244	0	244
$FMNH_2$	—36[3]	217	211
NADH + $FMNH_2$	129	112	241

[1] Computed from corrected absorbancy changes at 340 and 450 mμ.

[2] Computed from chemical assay for each reaction mixture.

[3] Computed from absorbancy change at 340 mμ due to oxidation of $FMNH_2$.

and Jagendorf (1) and Losada *et al.* (15) have shown that the NADP:reductase was also inhibited by CMB. Initial experiments with a crude nitrate reductase extract from corn tissue demonstrated that addition of 0.1 mM CMB to the regular assay mixtures inhibited (85–90 % loss of activity) the NADH-dependent reduction but was without effect on the $FMNH_2$-dependent activity. Since a scheme for mechanism of action of nitrate reductase (10) suggests that the enzyme has 3 sites for entry of electrons, these data on differential inhibition suggest that electrons from NADH and $FMNH_2$ enter at different sites.

Subsequent experiments not only verified the sulfhydryl requirement of the NADH-dependent reduction, but also demonstrated that $FMNH_2$:nitrate reductase activity was sulfhydryl dependent, although to a lesser degree. These statements are supported by the data of tables IV and V. It is also evident from the data in table V and figure 4 that the

Table IV. *Effect of Removal of Cysteine on NADH: and $FMNH_2$:Nitrate Reductase Activity*

A standard crude extract[1] from corn leaves was divided equally and passed through separate DEAE columns to remove ferredoxin. One column was pre-equilibrated with phosphate buffer (25 mM) only and the other with phosphate buffer (25 mM) plus cysteine (1.0 mM). Nitrate reductase did not adhere to the column, and passed directly through. The effluent from the latter column was then applied to a Sephadex G-50 column equilibrated with only phosphate buffer (0.025 M), to remove the cysteine from the extract. All columns were equilibrated at pH 7.5 and standard assays were used throughout.

Steps	Nitrate reductase activity mμmoles NO_2^- produced/0.2 ml enz/15 min $FMNH_2$	NADH	Ratio $FMNH_2$:NADH
1 Original extract	111.8	107.1	1.0
2 DEAE Column–PO_4 only[2]	93.5	64.2	1.5
3 DEAE Column–PO_4 plus cysteine	94.4	88.4	1.1
4 Sephadex G-50 column	30.7	1.8	17.0
5 Effluent from 4 plus 15 mM cysteine	76.5	65.6	1.2

[1] Homogenizing media was 10 mM with respect to cysteine.
[2] Discarded after assay.

Table V. *Influence of Time, Temperature, and Sulfhydryl Level on Nitrate Reductase Stability with NADH and $FMNH_2$ as Electron Donors*

Three comparable extracts were prepared from a composite sample of 10-day old greenhouse-grown corn leaves. The extraction media for the 3 extracts contained 10 mM, 0.1 mM, and no cysteine, respectively. Each extract was then subdivided into 3 equal fractions and placed at 0°, 28°, or 40°, respectively. Nitrate reductase activity was assayed at frequent intervals by standard assay. Each value represents the average of 2 samples.

Temperatures	0°		28°		40°	
Electron donor	NADH	$FMNH_2$	NADH	$FMNH_2$	NADH	$FMNH_2$
Extracted with 10 mM cysteine			*% of Initial*			
Initial	100	100	100	100	100	100
2 hr	102	97	37	95	0	32
6 hr	99	94	3	67	0	3
10 hr	93	101	4	47	0	0
24 hr	43	109	0	11	0	0
Extracted with 0.1 mM cysteine						
Initial	100 (2)[1]	100 (39)[1]	100	100	100	100
2 hr	0	90	0	62	0	3
6 hr	0	83	...	...	0	0
10 hr	0	70	...	...	0	0
24 hr	0	55	...	...	0	0
Extracted with no cysteine						
Initial	100 (0.4)[1]	100 (28)[1]	100	100	100	100
2 hr	0	100	0	54	0	0
6 hr	0	94	0	27	0	0
10 hr	0	80	0	20	0	0
24 hr	0	52	0	0	0	0

[1] Percent of initial extract in comparison to level obtained with 10 mM cysteine.

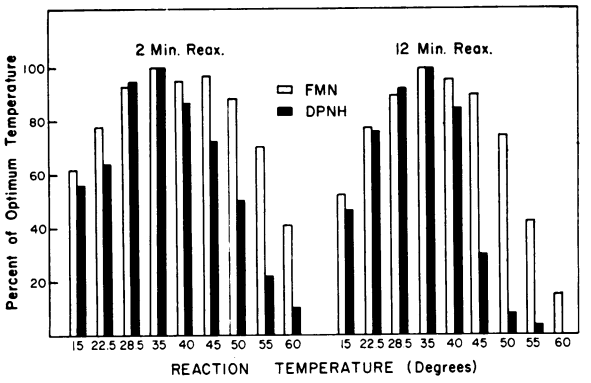

FIG. 4. The effect of reaction temperature on $FMNH_2$: and NADH:nitrate reductase activities over 2 and 12 minute reaction periods. A partially purified enzyme from corn (0.09 mg per assay) was used (see Methods). Standard $FMNH_2$: and NADH:nitrate reductase assays were used except for the variation in reaction period. The 100 % levels at 2 and 12 minutes represent 19 and 111 mμmoles NO_2^- formed.

NADH-dependent activity *in vitro* is more heat labile than the $FMNH_2$-dependent nitrate reduction. This is apparently due to the more sensitive −SH group associated with the NADH-dependent activity as the higher levels of exogenous cysteine afforded some increase in stability (table V). Although the optimum reaction temperature for nitrate reduction was 35° with both cofactors (fig 4), the NADH-activity decreased more rapidly than $FMNH_2$-activity at temperatures above 40°.

These experiments would suggest the existence of 2 enzymes, especially the apparent loss of NADH-activity during removal of cysteine (step 4, table IV), had it not been possible to effect an almost complete recovery of equal amounts of NADH- and $FMNH_2$-activities by addition of exogenous cysteine (step 5). The differential sensitivity of NADH- and $FMNH_2$-dependent nitrate reduction, and NAD:reductase to various concentrations of CMB and reversal of CMB inhibition by cysteine was investigated as a means of determining the relationship of NAD:reductase to nitrate reduction. Because exogenous sulfhydryl agents (*e.g.*, cysteine), which must be added to extract and stabilize NADH:nitrate reductase obtained from corn and spinach tissue, interfere with the DCIP assay of NAD:reductase, marrow cotyledons were used for the following work. Active nitrate reductase can be extracted from this tissue without the addition of sulfhydryl protective reagents, although occasionally the $FMNH_2$-dependent activity exceeds that of NADH when no exogenous sulfhydryl compounds are added during extraction.

The data of table VI show the relative level of sensitivity of $FMNH_2$: and NADH:nitrate reductase, and NAD:reductase to CMB. The observation that 5 μM CMB eliminates 98 % of the NAD:reductase activity and 100 % of the NADH:nitrate reductase activity with little (10 %) impairment of the $FMNH_2$ activity establishes that the NAD:reductase is not a requisite for $FMNH_2$-dependent nitrate reduction.

The addition of cysteine not only prevented CMB inhibition of NADH:nitrate reductase, but enhanced (60 vs 49 % — table VI) the level of NADH:nitrate reductase of the non-inhibited control (presumably by reduction of the sulfhydryl group on the enzyme).

The instability of the NADH activity in the absence of sulfhydryl agents suggested that purifi-

Table VI. *The Inhibition of $FMNH_2$ and NADH:Nitrate Reductase and NAD:Reductase by Various Levels of p-Chloromercuribenzoate (CMB) and Protection by Cysteine*

A standard crude extract (but without cysteine) was made from 8-day old marrow cotyledons and assayed for nitrate reductase and NAD:reductase as described in Methods, except for the addition of CMB and cysteine as shown. The 100 % control value for nitrate reductase was based on the activity (25 mμmoles of NO_2^-, mg protein^{-1}, min^{-1}) obtained with $FMNH_2$. The 100 % control value of NAD:reductase was 70 mμmoles DCIP reduced, mg protein^{-1}, min^{-1}. Addition of cysteine just prior to or after (3 min) addition of CMB to the enzyme was equally effective.

Conc in reaction mix		Nitrate reductase activity		NAD:reductase
CMB	Cysteine	$FMNH_2$	NADH	DCIP
M	M	*% of control*	*% of control*	*% of control*
None	None	100	49[1]	100
1×10^{-4}	None	38	0	...
5×10^{-5}	None	44	0	...
1×10^{-5}	None	68	0	0
5×10^{-6}	None	89	0	2
1×10^{-6}	None	101	0	22
None	10^{-3}	97	60	...[2]
5×10^{-6}	10^{-3}	88	60	...
1×10^{-6}	10^{-3}	96	60	...

[1] The relatively low level of NADH activity and the reactivation by cysteine is attributed to the fact that this preparation was frozen and stored at −30° prior to use. Other experiments with fresh extracts show the NADH activity to vary from 80 to 100 % of the $FMNH_2$ activity.

[2] Presence of cysteine prevented assay with DCIP.

Table VII. *Effect of Removal and Addition of Exogenous Cysteine during Purification on the Activities of NADH: and $FMNH_2$:Nitrate Reductase and NAD:Reductase*

The enzyme was extracted and purified from cotyledons of 10-day old marrow seedlings by standard procedures (see experimental methods), except for the omission of cysteine from steps 1 through 6 and the addition of 2 DEAE columns. The initial DEAE column (4 × 12 cm) was equilibrated with 25 mM tris, pH 7.5. After the enzyme preparation was placed onto the column, it was washed with a linear tris gradient (25 mM-150 mM, pH 7.5, 600 ml total volume). Nitrate reductase was then eluted from the column using a linear 0.15–0.8 M tris gradient (pH 7.5, 450 ml). The 5 best ml fractions were composited and diluted with 3 volumes of deionized water (0°) to lower the tris concentration in order that nitrate reductase would adhere to the second DEAE column (2 × 10 cm). Elution from the second column was accomplished using a linear 0.12–0.8 M tris gradient (pH 7.5, 400 ml). The eluate (the best eight 20 ml fractions) was concentrated by $(NH_4)_2SO_4$ (0–45 %) precipitation, and the resulting precipitate collected by centrifugation and resuspended in 25 mM phosphate buffer (pH 7.5) prior to use in step 6.

Fraction	Protein per ml mg	Protein total mg	Enzyme activities mμmoles NO_2^- or DCIP, mg protein^{-1}, min^{-1} Nitrate reductase NADH	Nitrate reductase $FMNH_2$	NAD:reductase (DCIP)	Ratios $FMNH_2$: NADH	Ratios DCIP: NADH
1 Extract	7.4	3920	7.5	8.3	65.0	1.1	8.7
2 $(NH_4)_2SO_4$ (22–42 %)	8.2	820	27.5	30.3	103.0	1.1	3.8
3 Eluate-from DEAE column	1.2	120	90.7	118.0	190.0	1.3	2.1
4 Eluate-from DEAE column	0.5	80	98.7	103.7	217.0	1.1	2.2
5 $(NH_4)_2SO_4$ (0–45 %)	2.2	44	101.3	109.0	233.0	1.1	2.3
6 PyroPO_4 0.1 M-eluate from $CaPO_4$-gel column			232.0	330.0	604.0	1.4	2.6
7 Eluate-from step 6 plus cysteine 5 mM	...	...	330.0	369.0	...	1.1	...

cation conducted without exogenous cysteine might achieve separation of NADH- and $FMNH_2$-activities. In the absence of cysteine it was also possible to measure the NAD:reductase activity at each stage of purification. As shown in table VII, separation of NADH- and $FMNH_2$-activities from marrow cotyledons was not achieved. Addition of 5 mM cysteine to the step 6 eluate (table VII) brought the NADH-activity to 330 mμmoles NO_2^-, mg protein^{-1}, min^{-1}, and a 1.1 ratio value of $FMNH_2$ to NADH activity. The loss of NAD:reductase during purification steps 1 and 2 (table VII) and the constancy of the activities of nitrate reductase to NAD:reductase obtained with steps 3 through 6 suggests that this NAD:reductase is a part of the NADH:nitrate reductase enzyme. The existence of other NAD: and NADP:reductases that could reduce DCIP but which apparently were not associated with nitrate reductase, was demonstrated as these enzymes could be separated from NADH-dependent nitrate reductase.

Since the NAD:reductase is the characteristic which distinguishes between NADH:nitrate reductase and $FMNH_2$:nitrate reductase it was thought that relative levels of NAD:reductase and NADH:nitrate reductase prior to and during induction with nitrate might clarify their relationship. Initial measurements established that extracts from 8-day old cotyledons from marrow plants grown on a minus nitrate medium had high levels (103 mμmoles DCIP reduced, mg protein^{-1}, min^{-1}) of NAD:reductase but essentially no NADH:nitrate reductase. Comparable tissue was used for induction studies (cotyledons floated in phosphate buffer, 20 mM, pH 4 at 32° with and without 0.1 M nitrate) and levels of NAD:reductase (DCIP assay) and NADH:nitrate reductase were determined after 2, 4, and 6 hours. Adequate controls (no nitrate) were used to permit measurement of net induction

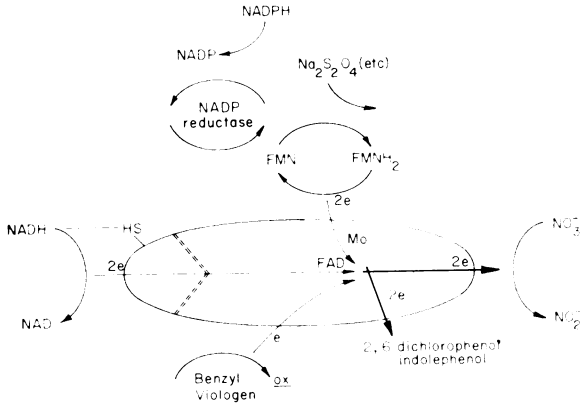

FIG. 5. A schematic representation of nitrate reductase showing electron flow patterns from NADH, NADPH via NADP:reductase and FMN, and chemical reductants ($Na_2S_2O_4$) via FMN. No distinction is implied between benzyl viologen and $FMNH_2$ for electron entry sites.

of both activities. The relative rates of increase for NAD:reductase, computed with the 2 hour measurements set at 100 % were 160 and 238 % (avg of 6 samples) at the 4 and 6 hour samplings, respectively. The relative rates of net induction (corrections made for initial level of activity) of the 2 activities were comparable as shown by range in ratio values (NAD:reductase/NADH:nitrate reductase) of 2.65, 2.50, and 2.32 (avg of 6 samples) after 2, 4, and 6 hours of induction, respectively. These ratio values are also comparable to those obtained with partially purified nitrate reductase preparations (table VII).

The concurrent appearance of both activities during induction with nitrate indicates that NADH: nitrate reductase is either a single protein or that synthesis of a specific NAD:reductase is required by the molybdoprotein for nitrate reduction. Since a relatively constant level of NAD:reductase activity remains associated with nitrate reductase during the final stages of purification, and this same relative level is obtained during induction, it is implied that NADH:nitrate reductase is a single protein moiety rather than 2 separate enzymes coupled by an unidentified cofactor. However, it is possible that 2 or more tightly bound subunits are involved and could not be resolved with the techniques employed.

A Model Structure for Nitrate Reductase. The diagram (fig 5) is patterned after the scheme used by Hewitt and Nicholas (10) in discussing mechanism of action of nitrate reductase. However, there are some major differences, namely: A) NADH is listed as the sole pyridine nucleotide electron donor based on the work of Beevers *et al.* (2); B) free $FMNH_2$ can serve as electron donor for nitrate reductase regardless of its mode of reduction, *i.e.*, chemically or enzymatically; and C) benzyl viologen is shown as an electron donor.

No attempts were made to determine whether $FMNH_2$ passes its electrons via FAD to Mo or directly to Mo. It was shown that when oxidized DCIP was added to a standard nitrate reducing system, the dye accepted the electrons from NADH and excluded the reduction of nitrate. It does not appear that the DCIP accepts electrons from molybdenum as DCIP can still be reduced when nitrate reduction is blocked by cyanide.

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