

Purification and Kinetics of Higher Plant NADH:Nitrate Reductase¹

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ABSTRACT

Squash cotyledon (*Cucurbita pepo* L.) NADH:nitrate reductase (NR) was purified 150-fold with 50% recovery by a single step procedure based on the affinity of the NR for blue-Sepharose. Blue-Sepharose, which is prepared by direct coupling of Cibacron blue to Sepharose, appears to bind squash NR at the NADH site. The NR can be purified in 2 to 3 hours to a specific activity of 2 μmol of NADH oxidized/minute \cdot milligram of protein. Corn (*Zea mays* L.) leaf NR was also purified to a specific activity of 6.9 μmol of NADH oxidized/minute \cdot milligram of protein using a blue-Sepharose affinity step. The blue-Sepharose method offers the advantages of a rapid purification of plant NR to a high specific activity with reasonable recovery of total activity.

The kinetic mechanism of higher plant NR was investigated using these highly purified squash and corn NR preparations. Based on initial velocity and product inhibition studies utilizing both enzymes, a two-site ping-pong mechanism is proposed for NR. This kinetic mechanism incorporates the concept of the reduced NR transferring electrons from the NADH site to a physically separated nitrate site.

The study of the biochemistry of higher plant NADH:nitrate reductase (NR)² has been retarded by the difficulty of obtaining the homogeneous enzyme. NR was first purified over 2 decades ago (10), but no purification scheme has been published which yields a plant NR highly purified with substantial recovery of total activity (14). In addition, NR is found to be labile after extraction and the absence of rapid and specific methods for purification results in large losses of enzyme activity (12). Affinity chromatography, with a bound ligand specific for NR, would appear to provide a means for rapid purification, which might also serve to concentrate the NR from the crude extract. Solomonson (27) used blue dextran-Sepharose as an affinity medium for purification of *Chlorella* NR to homogeneity. Application of higher plant NR to blue dextran-Sepharose results in a large percentage of apparent nonspecific binding and only partially achieves the results found for *Chlorella* NR (4). Other affinity purification techniques provide only partial purification of higher plant NR and the recovery of the NR is low (13, 20). Affinity chromatography has been used in combination with conventional techniques to purify NR to the highest reported specific activities but yields were low (11, 20). We describe here an affinity procedure

using blue-Sepharose for purification of higher plant NR. NR appears to be specifically bound to blue-Sepharose and can be recovered in high yields.

We have used the NR purified by this blue-Sepharose method from the extracts of corn leaf and squash cotyledons to investigate the kinetic mechanism of NR. Although little direct biochemical information exists to describe the components of higher plant NR, it is generally accepted to be of large molecular size (200,000-500,000 daltons), and to contain heme-iron, FAD, and molybdenum (14). These components appear to act in enzymic catalysis by NR as electron carriers in order to transfer electrons from the NADH reaction site to the nitrate reduction site (1). NR is considered to consist of two half-reactions: (a) NADH diaphorase and (b) reduced flavin nitrate reductase (14). These two half-reactions can be assayed independently from NADH reduction of nitrate and co-purify with the NADH:NR (14, 24). The half-reactions can be distinguished by differential inhibition or denaturation (5, 8). It appears that the NADH reaction site and the nitrate-reducing site of NR are physically separated and are probably located on different polypeptide chains.

Reduction of the NR electron carriers by NADH in the absence of an electron acceptor has been shown (14, 20). Considerable interest has centered on the reduction of NR as a mechanism for cellular regulation of NR (17). The reduced NR is inactivated but can be reactivated by oxidation with ferricyanide (21).

NR appears to be oxidized in the resting state and the electron carrier components of the enzyme become reduced during transfer of electrons from the NADH reaction site to the nitrate-reducing site. We describe here a kinetic mechanism for NR which incorporates the concepts of physically separated substrate sites which are connected by the reduction of electron transport components of NR.

MATERIALS AND METHODS

Plant Material. Squash (*Cucurbita pepo* L. cv. Buttercup) seeds were grown for 10 days in Vermiculite before harvest of the cotyledons. Corn (*Zea mays* L. cv. XL-81) seeds were germinated in Vermiculite and grown for 8 days before the leaves were harvested. All plants were subirrigated every other day with Hoagland solution fortified with 50 mM KNO₃ and 1 mM (NH₄)₂SO₄. The growth chamber was maintained on 14-hr day and 10-hr night at 27 C day and 24 C night.

Purification of NR. One hundred g of squash cotyledons were blended in 100 ml of 100 mM K-phosphate (pH 7.5), 1 mM EDTA, and 1 mM cysteine, with 20 g of PVP. The extract was filtered through Miracloth and centrifuged at 30,000g for 15 min. Twenty-five g of blue-Sepharose stirred gently with the supernatant solution for 1 hr at 4 C. The blue-Sepharose was recovered by filtration and washed with extraction buffer. After the blue-Sepharose was packed into a 2.5-cm-diameter column, the NR activity was eluted using a modification of the procedure described for soybean NRs

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² Abbreviations: NR: nitrate reductase; FAD: flavin adenine dinucleotide.

eluted from blue dextran-Sepharose (3). For elution of squash NR the extraction buffer was used in combination with 0.1 mM NADH. The NR activity fractions were pooled and concentrated by precipitation with 45% saturated ammonium sulfate. The concentrated NR was desalted on Sephadex G-25 in 100 mM K-phosphate (pH 7.5), 5 mM EDTA, and 1 mM cysteine.

For purification of corn NR, 200 g of leaves were blended in 400 ml of 200 mM K-phosphate (pH 7.5), 5 mM EDTA, and 10 mM cysteine with 20 g of PVP. After filtration, 0.1 volume of 1% (w/v) protamine sulfate solution containing 6 mg of Tris/ml of water was added and the extract was centrifuged. The supernatant was adjusted to 45% saturation with solid ammonium sulfate and the precipitate collected by centrifugation. The pellet was dissolved in 80 ml of 10 mM K-phosphate (pH 7.5), 1 mM EDTA, and 1 mM cysteine. The NR was further purified on blue-Sepharose as described above for squash extracts except 10 mM phosphate buffer was used for all procedures. The pooled and concentrated NR was desalted in the same manner as the squash NR.

Synthesis of Blue-Sepharose. Cibacron blue F3GA (Polysciences, Inc.) was directly coupled to Sepharose CL 4B (Pharmacia Fine Chemicals) to make blue-Sepharose (2).

NR Assays. NR assays using the colorimetric determination of nitrite were done in 25 mM K-phosphate (pH 7.5), 10 mM KNO₃, 0.1 mM NADH, and either 5 mM cysteine for corn extracts or 1 mM cysteine for squash extracts (23). NR assays for kinetic experiments were done in 23 to 28 mM K-phosphate (pH 7.5), 0.7 mM EDTA, and 0.7 mM cysteine by measuring the decrease in 340 nm *A* using a 1-cm cuvette and a Gilford 2400 spectrophotometer with a scale expander. The concentrations of substrates were varied from 0.03 to 5 mM KNO₃ and from 0.004 to 0.11 mM NADH. Initial velocity experiments were analyzed both by Lineweaver-Burk double reciprocal plots using linear regression and by the Eisenthal-Cornish-Bowden direct linear plot using an APL program for location of median intersections (26). Product inhibition studies were done in 1, 2, and 3 mM KNO₂ and in 0.5 and 0.75 mM NAD⁺. Kinetic studies using Cibacron blue F3GA as an inhibitor of NR were done with 6 and 14 nM concentrations of the dye (30). One unit of NR is defined as the amount of enzyme required either to produce 1 μmol of nitrite/min or to consume 1 μmol of NADH/min.

Protein Assays. Protein determinations were done by modifications of either the method of Lowry *et al.* (18) or the method of Sedmak and Grossberg (25). BSA (Sigma fraction V) was used as the standard protein and the two methods gave similar results.

Chemicals. All biochemicals were obtained from Sigma Chemical Company. Preweighed vials of the disodium salt of NADH (Sigma grade III) were used for kinetic experiments. NAD⁺ with Sigma grade III. Enzyme grade ammonium sulfate was obtained from Sigma. AnalaR analytical reagent grade potassium dihydrogen phosphate, EDTA disodium salt, potassium nitrate, and potassium hydroxide were obtained from BDH Chemicals Ltd. Potassium nitrite was analytical reagent grade obtained from Mallinckrodt Chemical Works. Deionized H₂O was used for preparation of all solutions.

RESULTS AND DISCUSSION

Purification of NR. Since higher plant NR binds poorly to blue dextran-Sepharose, the blue chromophore without the dextran could be a more specific ligand for affinity chromatography (4, 30). This chromophore, Cibacron blue, is a competitive inhibitor when NADH was varied using corn NR at fixed nitrate ($K_i = 3$ μM Cibacron blue). Blue dextran was also a competitive inhibitor of NADH but with a K_i of 18 μM. Cibacron blue can be directly coupled to Sepharose without need for cyanogen bromide activation of the gel (2). Essentially all of the NR activity of squash cotyledon extracts binds to blue-Sepharose and is not eluted by washing with extraction buffer. NR activity is eluted both with

NADH and KNO₃ (Fig. 1). NADH elutes the largest quantity of squash NR with a maximum specific activity of 12 units/mg of protein. In Table I, the results of six purifications of squash NR are summarized. The results of blue-Sepharose purification of corn NR are similar to those for squash NR, however, the bulk of the NR activity is eluted with nitrate. The corn NR eluted with NADH was labile and no attempt was made to stabilize this NR activity. In Table II, the results of a typical purification of corn NR are summarized.

Squash and corn NRs have been previously purified to specific activities of 0.2 to 0.7 units/mg of protein with recoveries of 20% or less (22, 24). The only reports showing higher specific activities for plant NR than those reported here are multistep procedures with recoveries of 10% or less (11, 20). One advantage of the blue-Sepharose method over these multistep procedures is the preparation of a high specific activity enzyme in 2 to 3 hr after extraction. Moreover, since the NR is obtained in high yield and can be concentrated by ammonium sulfate precipitation, an ample quantity of stable NR can be obtained for study of the enzyme's properties. This rapid purification of the NR minimizes the exposure of the enzyme to conditions that could alter the conformation.

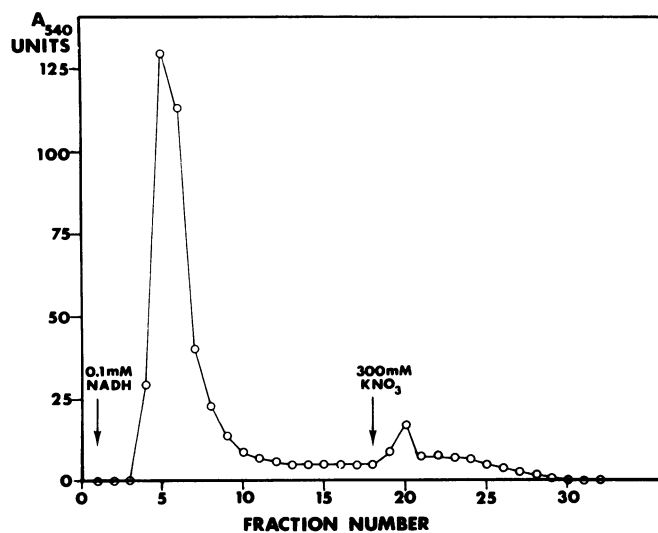


FIG. 1. Elution of squash cotyledon NR from blue-Sepharose. Ten-ml fractions were collected and assayed for NR activity by the colorimetric method (23).

Table I. Purification of Squash NR

The results are the average of six experiments

Procedure	Units	Specific Activity	Recovery	Purified	
	total	mg prot	units/mg	%	fold
Homogenate	31	2600	0.012	100	1
Blue-Sepharose NADH Peak	9.3	5	1.9	48	154

Table II. Purification of Corn NR

Procedure	Units	Specific Activity	Recovery	Purified	
	total	mg prot	units/mg	%	fold
Homogenate	11	990	0.011	100	1
Protamine Sulfate Supernatant	9.2	490	0.019	84	1.7
Ammonium Sulfate Fraction	9.9	180	0.055	90	5
Blue-Sepharose NADH Peak	2.4	0.35	6.9	22	630
KNO ₃ Peak	4.8	5.4	0.89	44	80

Properties of NR. The properties of blue-Sepharose-purified NR are similar to those previously described for higher plant NR (1, 14). The pH optimum is 7.5 and the NRs are inhibited by both *p*-hydroxymercuribenzoate and cyanide. In determination of the nitrate apparent K_m for both corn and squash NR, the double reciprocal plots were found to be biphasic (Fig. 2). Since corn leaf has been reported to have only a single species of NR (29) and we found no evidence to suggest the presence of two forms of NR in these preparations, the biphasic response to nitrate is considered

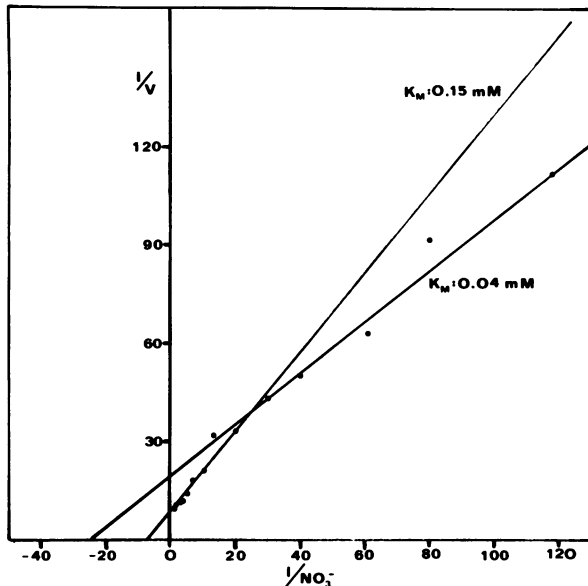


FIG. 2. Double reciprocal plots of the initial NR reaction velocity versus the nitrate concentration at 0.2 mM NADH. Nitrate concentration was varied from 0.004 to 1.2 mM. The NR assays were done by the colorimetric method (23). The K_m values (apparent) were derived by direct linear plots (26).

to be a property of the highly purified NR. Crude NR from spinach leaves has been reported to have a biphasic response to nitrate and freezing and thawing of the enzyme preparation reduced the nitrate kinetics to one phase (9). The biphasic kinetics data suggest that NR has negative cooperativity toward nitrate (16). The molecular structure of higher plant NR is yet to be determined and the number of nitrate-binding sites is not known. Inasmuch as the biphasic response could result from causes other than subunit interaction (28), further study of the NR is required before an adequate explanation can be offered.

NR Kinetics. The kinetic mechanism of an enzyme is an important component leading to a model of the mechanism of action of the enzyme. Apparent K_m values for the substrates of NR have been determined but little is known about the interaction of the substrates (1, 14). "Aged" crude spinach NR was reported to have a ping-pong kinetic mechanism. However, the authors did not include a study of product inhibition which is an important component of a kinetic study (9). We have investigated the kinetics of NR using both initial velocity and product inhibition studies. The preparations of NR used for the kinetic studies were purified by the blue-Sepharose method and had specific activities of at least 2 and 0.5 units/mg of protein for squash and corn, respectively.

Since available instrumentation was not sufficiently sensitive for determination of accurate rates of reaction at low substrate concentrations, lower limits of 30 μM nitrate and 4 μM NADH were used in all experiments. Since low substrate concentrations were not used, the double reciprocal plots were monophasic straight lines. The results of initial velocity experiments for squash NR are shown in Figure 3 with NADH varied at fixed nitrate concentrations and in Figure 4 with nitrate varied at fixed NADH concentrations. Replots of $(V_{\text{max}} \text{ apparent})^{-1}$ versus (nonvaried substrate concentration) $^{-1}$ were used to obtain K_m values and the V_{max} (Fig. 5). Similar results were obtained from a replot of $(K_m \text{ apparent})^{-1}$ versus (nonvaried substrate concentration) $^{-1}$. Corn NR gave initial velocity double reciprocal plots with parallel lines identical in form to those shown in Figures 3 and 4. Replots of

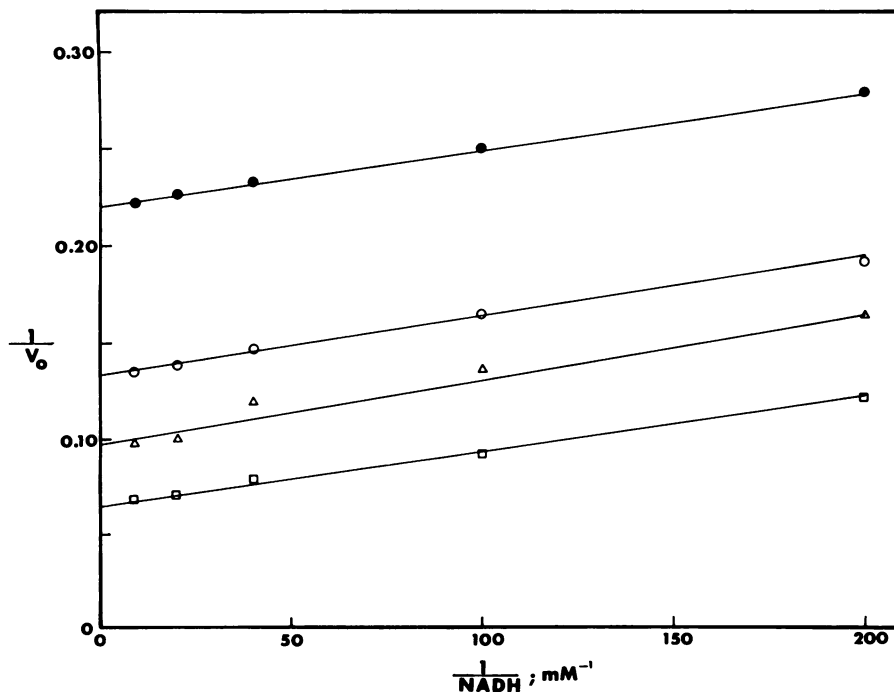


FIG. 3. Double reciprocal plots of the initial NR reaction velocity versus NADH concentration at fixed concentrations of nitrate. The fixed mM concentrations of nitrate were: 0.05 (●—●), 0.10 (○—○), 0.17 (△—△), and 0.67 (□—□).

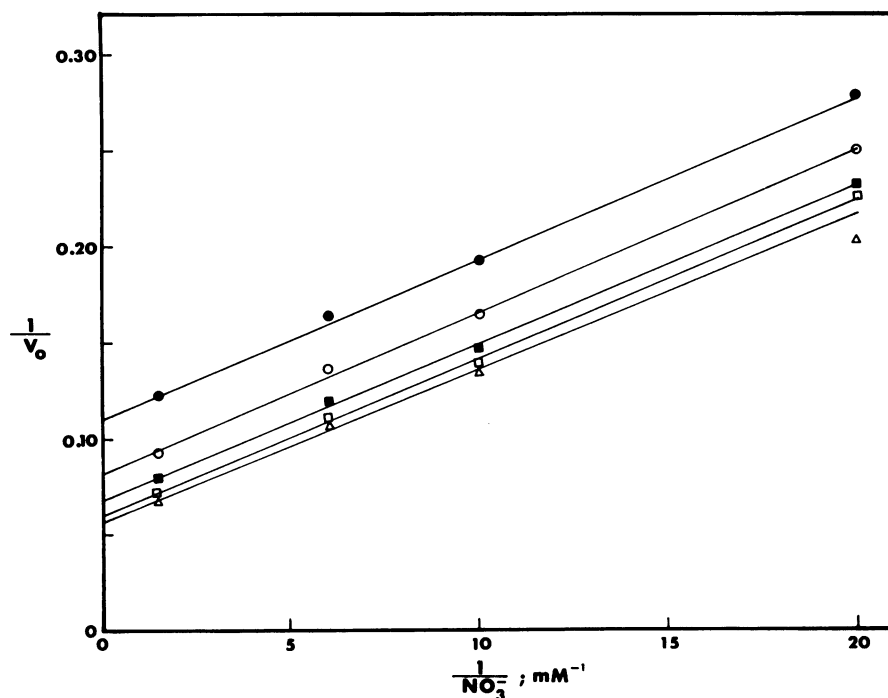


FIG. 4. Double reciprocal plots of the initial NR reaction velocity *versus* the nitrate concentration at fixed concentrations of NADH. The fixed mM concentrations of NADH were: 0.005 (●—●), 0.010 (○—○), 0.025 (■—■), 0.050 (□—□), and 0.11 (△—△).

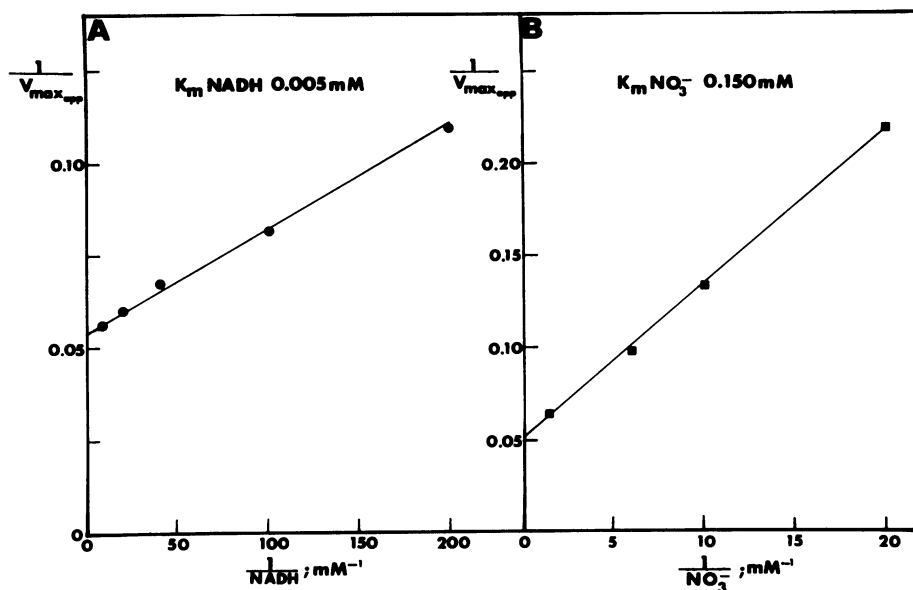


FIG. 5. Derivative double reciprocal plots of maximum velocity (apparent) *versus* nonvaried substrate concentration. Plot A was derived from Figure 5 and was used to calculate the K_m for NADH. Plot B was derived from Figure 4 and was used to calculate the K_m for nitrate.

$(V_{\max \text{ apparent}})^{-1}$ *versus* (nonvaried substrate concentration) $^{-1}$ gave K_m values of 3 μM NADH and 70 μM nitrate.

Product inhibition patterns were the same for both squash and corn NR. NAD^+ shows competitive inhibition toward NADH at both subsaturating and saturating nitrate concentrations. NAD^+ was a mixed type inhibitor toward nitrate at subsaturating concentrations of NADH. Nitrite was a competitive inhibitor of nitrate at both subsaturating and saturating concentrations of NADH. Nitrite was a mixed type inhibitor of NADH at subsaturating concentrations of nitrate. Table III summarizes the product inhibition patterns and K_i values for squash and corn NR.

The parallel lines in the double reciprocal plots of the initial velocity experiments indicate the kinetic mechanism is of the ping-

pong type (6). The ping-pong mechanism for a two-substrate reaction has the general characteristic of a modification of the enzyme by the first substrate and the departure of the first product before binding of the second substrate. For NR the product inhibition patterns are not consistent with the mechanism being a standard ping-pong type. The product inhibition pattern for NR is similar to that found for transcarboxylase (19) and several other enzymes (15). This mechanism can be described as a two-site ping-pong type where the enzyme has physically separated sites for binding of the substrates and catalysis involves physical transfer of an entity from one substrate site to the other substrate site. For NR, previous reports substantiate the existence of physically separate binding sites for NADH and nitrate (1, 14) and that flavin,

Table III. Product Inhibition Pattern for Squash and Corn NR

Product	NADH Varied				Nitrate Varied			
	Subsat. Nitrate	K_i^1	Sat. Nitrate	K_i	Subsat. NADH	K_i	Sat. NADH	K_i
NAD ⁺	C ²	A B 0.83 0.26	C	A B 1.3 0.15	MT	A B 5.0 1.0	---	A B --- ---
Nitrite	M ²	3.0 1.2	---	---	C	0.25 0.33	C	0.9 1.4

¹ K_i 's are apparent product inhibition constants expressed as mM. Column A values are for squash NR and column B values are for corn NR.

²C represents competitive inhibition and MT represents mixed type inhibition.

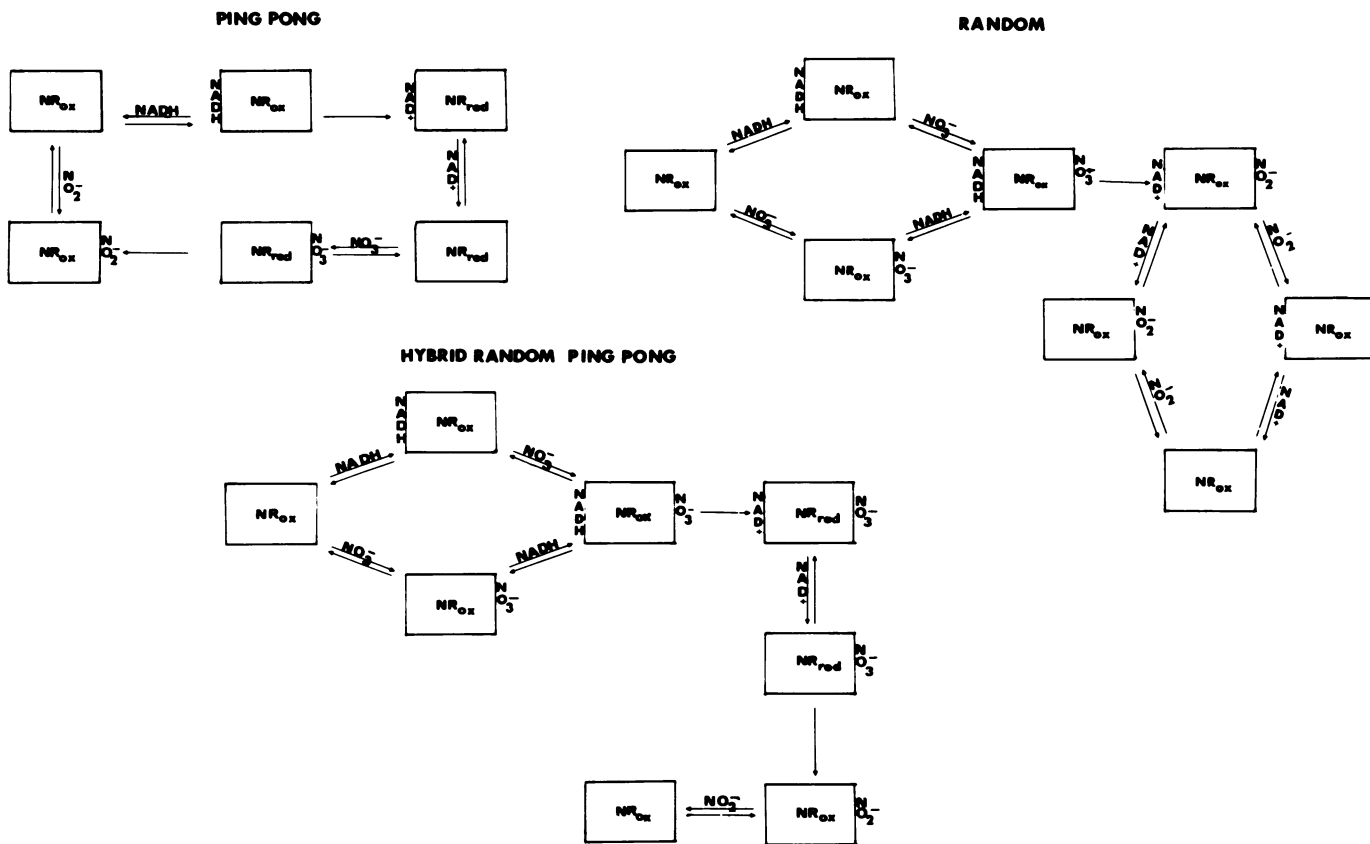


FIG. 6. Kinetic model proposed for the two-site ping-pong mechanism of higher plant NADH:NR.

heme-iron, and molybdenum are components of enzyme involved in transfer of electrons from the NADH site to the nitrate site (1, 14). NR appears to be oxidized initially and is reduced by NADH prior to donating electrons to nitrate.

In order to obtain the product inhibition patterns of nitrite as a competitive inhibitor of nitrate and NAD⁺ competitive with NADH, one must assume that all of these components bind to the oxidized form of the NR. The random order of substrate binding of the NR mechanism is shown by these product inhibition results but requires confirmation by binding studies. These kinetic results indicate that NR catalysis can occur by one of several pathways depending on the order of events of substrate binding and reduction of NR. Figure 6 shows three reaction pathways that occur during the NR catalysis, the actual mechanism being a composite of these three. One sequence is the standard ping-pong mechanism where nitrate binds to the reduced enzyme after release of NAD⁺. Another is a completely random addition of substrates and release of products. The third is a hybrid where random binding of substrates leads to the reduced NR and release of NAD⁺ before nitrate accepts the electrons. When both the rapid equilibrium and steady-state assumptions are applied, a rate equation for this

mechanism can be derived which is identical to that of the standard ping-pong mechanism in the absence of products (7, 26). Studies of the kinetics of NR when reduced prior to nitrate addition, the kinetics of the NADH diaphorase reaction, and the dye-mediated nitrate reductase are currently being done and may provide additional insight into the kinetic mechanism of NR.

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