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Hysteretic Behavior of Nitrate Reductase

EVIDENCE OF AN ALLOSTERIC BINDING SITE FOR REDUCED PYRIDINE NUCLEOTIDES*

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In the absence of NADH, at 25 °C, partially purified NADH:nitrate reductase undergoes an approximately 50% reduction of its initial activity during 2 h. With the increase of inactivation, the NADH and nitrite concentration time curves become typical "sigmoidal," *i.e.* the reaction velocity of the nitrate reductase catalyzed reaction goes through a maximum before equilibrium is reached. About 80% of the original activity of nitrate reductase is restored when the enzyme is incubated for 2 min with 200 μM NADH or NADPH. Also other NADH substrate analogues have similar effects in restoring the lost activity. After incubation with the reduced pyridine nucleotides, the sigmoidal appearance of the NADH concentration time curve disappears almost completely. Despite the fact that NADPH increases the activity of the enzyme, NADPH does not show any competition with the NADH-binding site of nitrate reductase and does not produce nitrite in the absence of NADH. It is therefore concluded that there must be an additional allosteric site which binds either NADH or NADPH, or other pyridine nucleotides with the effect of increasing the activity of the enzyme. A kinetic model is presented which simulates the observed experimental findings.

NADH:nitrate reductase catalyzes the reduction of nitrate to nitrite.

$$NADH + H^+ + NO_3^- \xrightarrow{NR} NAD^+ + NO_2^- + H_2O \quad (R1)$$

Process R1 is the rate-limiting step in the assimilation of nitrate to organically bound nitrogen in higher plants and algae. NADH:nitrate reductase is a complex enzyme consisting of two identical monomers. Each monomer has three functional domains containing FAD, heme, and molybdenum pterin as prostetic redox groups (Caboche and Rouzé, 1990). NADH:nitrate reductase is regulated at the nucleic acid level (Caboche and Rouzé, 1990). However, fine tuning of the enzyme's activity at the protein level may also be of physiological importance (Solomonson and Barber, 1990; Lillo, 1991).

Most, if not all, nitrate reductase preparations from higher plants are unstable, and this could influence the conclusions drawn from kinetically and regulatory studies. The kinetic studies performed for NADH:nitrate reductase indicate a random order ternary complex mechanism for the Chlorella enzyme and a ping-pong mechanism for the squash, maize, and spinach enzyme (Solomonson and Barber, 1990). A kinetic study of the NADH inhibition pattern of maize nitrate reductase purified by monoclonal antibodies indicates a ternary complex mechanism contrary to the earlier concluded two-site ping-pong mechanism (Ruoff, 1990).

In this paper we present analyses of the concentration time plots of consumed NADH and produced nitrite ion for reaction R1. From experiments and related model calculations we provide evidence that nitrate reductase has an additional allosteric site that binds NADH, NADPH, or other substrate analogues and that upon binding increases the enzyme's activity.

MATERIALS AND METHODS

Cultivation of Plants—Squash (*Cucurbita maxima* L. cv. *Buttercup*) were grown in vermiculite in 30-cm pots. There were 30 seeds/pot. One liter of Hoagland solution No. 1 with 15 mM KNO_3 and no ammonium (Hoagland and Arnon, 1950) was added at sowing and 1 day before harvesting leaves. Leaves were harvested every second week from 4-8-week-old plants. The plants were grown at 28 °C under fluorescent lamps (Osram L 58 W 177), and a 16-h photoperiod.

Enzyme Preparation—Nitrate reductase was partially purified using Blue-Sepharose and hydroxylapatite (Redinbaugh and Campbell, 1981; Notton *et al.*, 1985). Leaves, 300 g, were homogenized in a Waring blender with 1 liter of 0.1 M potassium phosphate buffer, pH 8.0, 1 mM EDTA, 7 mM cysteine, 1 μM leupeptin, 1 μM FAD, 0.1 mM phenylmethylsulfonyl fluoride, and 30 g of polyvinylpyrrolidone. The macerate was filtered through one layer of Miracloth and two layers of cheesecloth, and centrifuged for 30 min at 12,000 rpm in a Sorvall SS-34 rotor (average 11,227 × *g*). The supernatant was stirred slowly with 70 g of Blue-Sepharose for 50 min and then collected by vacuum filtration. The Blue-Sepharose was then washed with 0.5 liter of extraction buffer without polyvinylpyrrolidone, and then with 1 liter of a second wash buffer: 25 mM potassium phosphate buffer, pH 7.5, containing 3.5 mM cysteine, 1 μM FAD, 1 μM leupeptin. Blue-Sepharose was poured into a column and nitrate reductase was eluted with 0.1 mM NADH dissolved in the second wash buffer.

Most active Blue-Sepharose fractions were collected and 1 g of hydroxylapatite (Bio-Rad) was added. After 20 min shaking, the hydroxylapatite was collected by centrifuging for 10 min at 10,000 rpm in a Sorvall SS-34 rotor (average 7,796 × *g*). The supernatant was discarded, and the hydroxylapatite was washed first with 40 ml of elution buffer and thereafter with 25 ml of 50 mM potassium phosphate buffer containing cysteine, FAD, and leupeptin as described above. Nitrate reductase was then eluted from the hydroxylapatite with 0.125 mM potassium phosphate and additives as described above. The enzyme was precipitated with $(NH_4)_2SO_4$ and desalted on a Sephadex G-25 column with 50 mM potassium phosphate buffer and 1 mM EDTA, pH 7.5. Recovery was about 25%, and the specific activity was 12 units/mg protein. Nitrate reductase was purified 800-fold during this procedure. Protein was measured with the Bio-Rad protein assay using bovine γ-globuline as a standard.

Nitrate reductase was assayed by spectrophotometric measurement at 540 nm the amount of nitrite produced as the diazo compound formed from sulfanilamide (Lillo, 1983). One unit of nitrate reductase activity is defined as the rate of 1 μmol of nitrite produced in 1 min.

Kinetic Experiments—Kinetic experiments were performed at 25 ± 1 °C. The NADH and nitrate solutions, as well as dilution buffer and enzyme, were stored in a thermostatted water bath at 25 ± 1 °C. Reaction rates were either measured point wise as the amount of produced nitrite ion during a given period of time, or continuously by following the consumption of NADH or other NADH analogues in a

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spectrophotometric cell at 340 nm. The reaction volume was 1000 μl.

Model Calculations—Simulation calculations were performed by integrating the chemical rate equations using the FORTRAN subroutine LSODE (Hindmarsh, 1980).

RESULTS

When assaying nitrate reductase immediately after desalting the enzyme preparation, a linear consumption of NADH and production of nitrite was observed. However, when the enzyme preparation was stored at room temperature and aliquots were taken out and tested, a lag in consumption of NADH and in the production of nitrite was observed (Fig. 1). This sigmoidal behavior appeared after about a half hour of storage and we observed it not only in hydroxylapatite preparations or fractions eluted directly from Blue-Sepharose, but also in preparations from monoclonal antibodies (Ruoff and Lillo, 1990).

The quotient between the initial reaction rate, V_0 (*i.e.* the reaction rate during the first half minute of the assay), and the most rapid reaction rate, V_{max} (*i.e.* the reaction rate after about 4 min of incubation, see Fig. 1*B*) was calculated to characterize the sigmoidal behavior. This quotient decreased as a function of time when the enzyme was stored at 25 °C. After about 1 h of storage, the initial reaction rate was only about half of the most rapid reaction rate observed during the assay time (the quotient was 0.5). After 5 h of storage this quotient was about 0.35 (Fig. 2).

When the assay was run for 6 min with 5 mM nitrate and 175 μM NADH, the reaction velocity increased during the assay period in accordance with Fig. 1, and all the NADH was consumed (Fig. 3, *curve 1*). However, when new NADH was added to the assay mixture, the NADH consumption was linear, and V_{max} was gained immediately (Fig. 3, *curve 2*).

Preincubation of the enzyme with 200 μM NADH almost abolished the lag. In some experiments, when the control had a quotient of 0.6, preincubation with NADH for 2 min was sufficient to abolish completely the lag. In other experiments, when the control was characterized by a lower quotient, the V_0/V_{max} was not brought completely back to 1 during 2 min of preincubation. However, the effect of preincubation was clear (Table I). Interestingly, preincubation with NADPH also abolished the lag. Lower concentrations of NAD(P)H, for example, 20 μM, also had a clear effect on the initial reaction rate (Table II).

Preincubation with various adenine compounds showed that ATP, benzyladenine, and NAD^+ did not abolish the lag. The NAD(P)H analogues, NHDH,[1] the reduced form of nic-

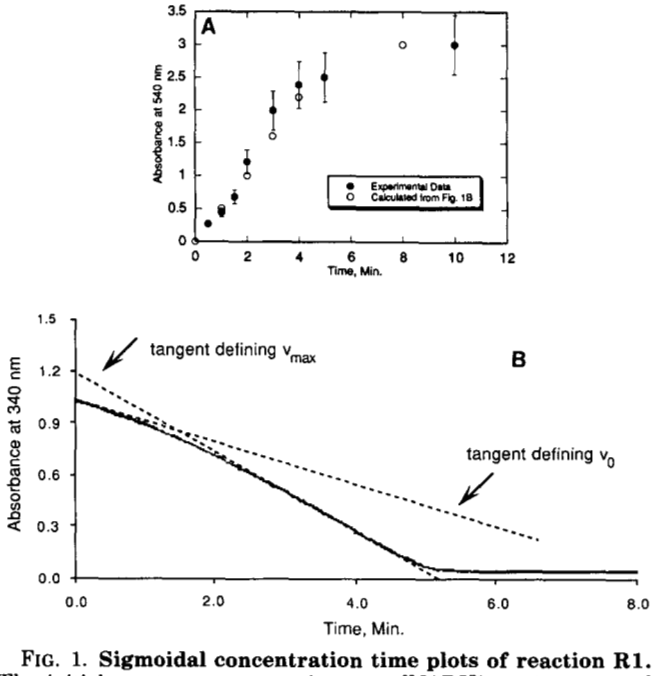

FIG. 1. **Sigmoidal concentration time plots of reaction R1.** The initial reactant concentrations are $[NADH]_0$ = 175 μM and $[NO_3^-]_0$ = 5 mM. *A* shows the amount of formed nitrite ion measured as the absorbance of the formed diazo-compound of sulfanilamide at 540 nm. *Error bars* of experimental data indicate an uncertainty of 15%. *Open circles* show calculated absorbances from the amount of consumed NADH (see *B*). *B* shows the concentration time plot of NADH measured at 340 nm. Tangents defining initial rate V_0 and maximum rate V_{max} are indicated.

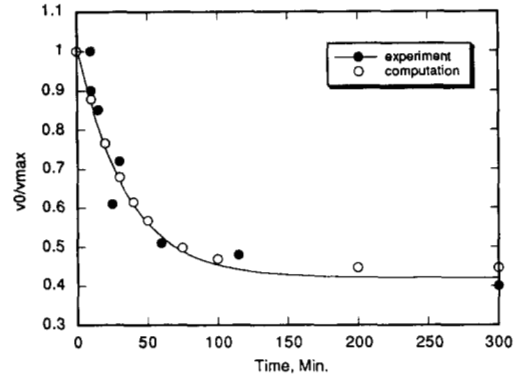

FIG. 2. **Experimental and computed V_0/V_{max} values as a function of storage time of freshly prepared and desalted nitrate reductase.**

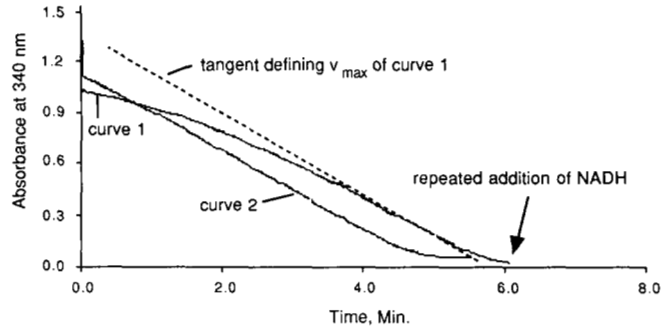

FIG. 3. **Concentration time plot of NADH measured at 340 nm.** *Curve 1* has the same initial reactant concentrations as in Fig. 1*B*. When all NADH has been consumed more NADH was added which resulted in *curve 2*.

TABLE I

Effect of various preincubation times with 200 μM NADH or NADPH on V_0/V_{max}

There were three parallels for each treatment. The standard deviation is given.

Preincubation time	V_0/V_{max}	
	Experiment	Computation
s		
0 NADH	0.47 ± 0.01	0.43
30 NADH	0.62 ± 0.04	0.55
60 NADH	0.70 ± 0.12	0.65
120 NADH	0.84 ± 0.06	0.80
240 NADH	0.88 ± 0.03	0.92
120 NADPH	0.80 ± 0.08	0.80
240 NADPH	0.88 ± 0.03	0.92

[1] The abbreviations used are: NHDH, reduced form of nicotinamide hypoxanthine dinucleotide; NHDPH, reduced form of nicotinamide hypoxanthine dinucleotide phosphate; 3-aADH, reduced form of 3-acetylpyridine adenine dinucleotide.

TABLE II

Effect of preincubating the enzyme with various concentrations of NAD(P)H for 2 min on V_0/V_{max}

There were three parallels for each treatment and the standard deviation is given.

[NAD(P)H]	V_0/V_{max}		
	NADH	NADPH	Computed
μM			
0	0.40 ± 0.01	0.39 ± 0.02	0.43
3	0.43 ± 0.02	0.46 ± 0.02	0.46
20	0.77 ± 0.01	0.59 ± 0.03	0.57
200	0.83 ± 0.03	0.76 ± 0.01	0.80

TABLE III

Effect of preincubation of the enzyme on the relative values of initial and most rapid reaction rate

The enzyme was preincubated for 2 min with 200 μM of various compounds before running the assay. The data given are the average of two separate experiments with standard deviation.

Compound tested	V_0/V_{max}
None (control)	0.50 ± 0.01
NAD^+	0.49 ± 0.01
ATP	0.45 ± 0.02
Benzyladenine	0.50 ± 0.01
NADH	0.89 ± 0.02
NADPH	0.85 ± 0.05
NHDH	0.88 ± 0.02
NHDPH	0.89 ± 0.02
3-aADH	0.88 ± 0.02

otinamide hypoxanthin dinucleotide, NHDPH, the reduced form of nicotinamide hypoxanthin dinucleotide phosphate, and 3-aADH, the reduced form of 3-acetyl pyridine adenine dinucleotide, were as efficient as NADH (Table III). All the tested NAD(P)H analogues could completely remove the lag when V_0/V_{max} was relatively high, about 0.65.

These various NAD(P)H analogues also reduced NO_3^- to NO_2^- in the presence of nitrate reductase. At 175 μM NAD(P)H analogues and 5 mM NO_3^-, the analogues gave activities relative to the NADH activity as follows: NADH 100%, NHDH 98%, NHDPH 33%, 3-aADH 26%, NADPH 2%. Consumption of NADPH could only be observed when high concentrations of enzyme (5 times the regular enzyme amount) were added to the assay mixture.

3-aADH at 200 μM was competitive with 20 μM NADH, and reduced the reaction rate to 40% of the control. However, NADPH and NHDPH at 200 μM did not influence the reaction velocity (Table IV). Subsaturating concentrations of NADH (5 and 10 μM) were tested across a range of NADPH concentrations (5–100 μM). However, no inhibition by NADPH was observed. This indicates that nitrate reductase has a second regulatory binding site for NAD(P)H and their analogues that is less selective with respect to the reduced pyridine nucleotides compared to the catalytic site.

Computational Results—In order to explain the increase of reaction velocity in Fig. 1, we assume that nitrate reductase occurs in two enzymatic forms: one active form E^*, and one less active (but still not inactive) form E. E is transformed to E^* when NAD(P)H or their analogues bind to the regulatory site.

$$E + \text{NAD(P)H} \underset{k_2}{\overset{k_1}{\rightleftarrows}} E[\text{NAD(P)H}]_{\text{regulatory site}} \quad \text{(A1)}$$

$$E[\text{NAD(P)H}]_{\text{regulatory site}} \xrightarrow{k_3} E^*[\text{NAD(P)H}]_{\text{regulatory site}} \quad \text{(A2)}$$

$$E^*[\text{NAD(P)H}]_{\text{regulatory site}} \underset{k_5}{\overset{k_4}{\rightleftarrows}} E^* + \text{NAD(P)H} \quad \text{(A3)}$$

TABLE IV

Initial reaction rate of nitrate reductase activity at various concentrations of NADH plus NADPH or NAD(P)H analogues

$[NO_3^-]_0$ = 5 mM.

[NADH]	[NADPH], [NHDPH], [3-aADH]	V_0
	μM	$\Delta A_{340}/min$[a]
200		0.082
0	200 (NADPH)	0.000
20		0.081
20	200 (3-aADH)	0.033
20	200 (NHDPH)	0.080
20	200 (NADPH)	0.081
10		0.060
10	10 (NADPH)	0.060
10	100 (NADPH)	0.060
5		0.039
5	10 (NADPH)	0.039
5	50 (NADPH)	0.039

[a] Change of absorbance per min recorded at 340 nm.

In the computations we assume that $k_1 = 1.0 \cdot 10^{10}$ M^{-1} min^{-1}, $k_2 = 8.0 \cdot 10^5$ min^{-1}, $k_3 = 0.6$ min^{-1}, $k_4 = 8.0 \cdot 10^5$ min^{-1}, $k_5 = 2.7 \cdot 10^{10}$ M^{-1} min^{-1}. In addition, we assume that activation also occurs for those enzyme species that have substrates bound at their catalytic sites. In the absence of NAD(P)H, E^* is converted to E.

$$E^* \xrightarrow{k_6} E \quad \text{(B1)}$$

The time scale of process B1 is indicated in Fig. 2. The decrease in V_0/V_{max} is kinetically well described when k_6 = 0.0342 min^{-1} (Fig. 2). In the computations the catalytic mechanism is simply described by processes C1–C3,

$$E + \text{NADH} \xrightarrow{k_7} E[\text{NADH}]_{\text{catalytic site}} \quad \text{(C1)}$$

$$E[\text{NADH}]_{\text{catalytic site}} + NO_3^- \xrightarrow{k_8} E[\text{NADH}, NO_3^-]_{\text{catalytic site}} \quad \text{(C2)}$$

$$E[\text{NADH}, NO_3^-]_{\text{catalytic site}} \xrightarrow{k_9} E + \text{products} \quad \text{(C3)}$$

where $k_7 = 1.0 \cdot 10^{10}$ M^{-1} min^{-1}, $k_8 = 2.8 \cdot 10^7$ min^{-1}, and $k_9 = 1.0 \cdot 10^3$ min^{-1}. In case E^* is catalytically active, the rate constant values of the reactions analogous to C1-C3 are increased by a factor of 2.7.

The experimental incubation data shown in Table I are well described by the model. Table I also contains the corresponding computed V_0/V_{max} values.

Table II compares computed V_0/V_{max} values with the corresponding experiments as a function of the amount of incubated NADH or NADPH.

In the case that all NADH has been consumed, and new NADH is added to the system, the reaction practically restarts with maximum velocity, but without the sigmoidal shape (Fig. 4). Also this is in good agreement with the corresponding experimental findings (Fig. 3).

DISCUSSION

Because NAD(P)H can bind to a site of nitrate reductase that is different from the catalytic site and increase the activity of the enzyme, nitrate reductase is an allosteric enzyme. The existence of an allosteric site in nitrate reductase has so far not been recognized.

Although the experiments clearly indicate the presence of an additional allosteric site in nitrate reductase, we so far cannot determine from the experiments the number of allosteric sites in relation to the catalytic sites. This is reflected by the simplicity of the kinetic model which treats nitrate reductase simply as a monomer where we have assumed that there is only one allosteric site present. However, despite the

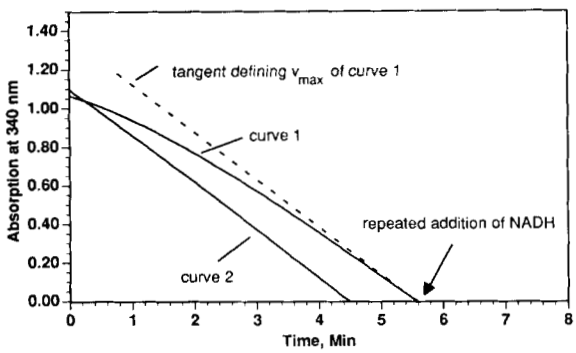

FIG. 4. **Simulation calculation of a system corresponding to that of Fig. 3.** Initial concentrations: $[NADH]_0 = 175\ \mu M$, $[NO_3^-]_0 = 5$ mM, $[E]_0 = 16$ nM (*curve 1*). When all NADH was consumed, the NADH concentration was set to 180 μM, and the calculation continued (*curve 2*).

simplicity of the considered model reactions, it appears that the slow transformation of enzyme species E to E^* is essential in order to explain the initial lag and the activation of nitrate reductase by reduced pyridine nucleotides.

Generally, several allosteric enzymes show sigmoidal velocity curves, *i.e.* the initial velocity as a function of initial concentration of the "activating" substrate has a typical "S-shape" (Segel, 1975). Sigmoidal velocity curves can be explained because there is a rapid equilibrium between the activating substrate and the allosteric sites binding the substrate. In addition to the increased rate due to the increased substrate concentration, there is also an increase of kinetically more active enzyme as a result of activating substrate molecules binding at the allosteric site. When the initial concentration of the (activating) substrate is increased, the initial reaction velocity increases exponentially. We could term this behavior "concentration allosteric." In the nitrate reductase system studied here, the more active enzyme species, E^*, are produced by a *slow* process that forms the more active enzyme species. This behavior could be called "time allosteric," because here the concentration time plots are sigmoidal, but not the velocity plots.

Enzymes that exist in two forms which have different activities and interconvert slowly between these forms due to various mechanisms are called hysteretic enzymes (Frieden, 1970; Neet and Ainslie, 1980). According to this terminology, nitrate reductase is a hysteretic enzyme. The slow allosteric interaction in nitrate reductase is only one example how conversion between enzymatic forms can occur.

There are a variety of enzymes that show analogous "lag behavior" as nitrate reductase. Frieden (1970) and Neet and Ainslie (1980) have provided lists of several enzymes that show a similar time lag behavior as nitrate reductase. Interestingly, like nitrate reductase, these hysteretic enzymes, for example, glyceraldehyde-3-phosphate dehydrogenase (Frieden, 1970), phosphofructokinase (Frieden, 1970), or phosphoenolpyruvate carboxylase (López-Pozos *et al.*, 1990), also appear important in the regulation of metabolic processes. On the other hand, the physiological significance of such hysteretic behaviors is still not understood.

In the case of nitrate reductase, the appearance of the time lag is not only found in squash, but was also observed for corn nitrate reductase that was purified with monoclonal antibodies (see Fig. 1 in Ruoff and Lillo (1990)). Pyridine nucleotides are important regulators of carbon metabolism in plants, for instance, through their effect on pyruvate dehydrogenase, glucose-phosphate dehydrogenase, and isocitrate dehydrogenase (Raymond *et al.*, 1987). Nitrogen metabolism has to be controlled in cooperation with carbon metabolism as pointed out by several authors (Kaiser and Brendle-Behnisch, 1991; Turpin and Weger, 1990). However, it is still not clear how this is achieved. Control by pyridine nucleotides could constitute part of this regulation. Clearly, physiological concentrations of NAD(P)H are in the range that would effect nitrate reductase activity (Bonzon *et al.*, 1983). Furthermore, the concentration of NADPH increases during the first 30 min when plants are transferred from darkness to light (Rao *et al.*, 1990). Although the results presented in this paper do not prove any physiological significance of NAD(P)H as a regulator of nitrate reductase activity, the light-modulated increase in NADPH could explain part of the positive light effect on nitrate reductase activity (Lillo, 1991). However, the physiological relevance of NAD(P)H as an allosteric regulator for nitrate reductase is not understood.

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