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NADH SUBSTRATE INHIBITION IN CORN LEAF NITRATE REDUCTASE: EVIDENCE FOR A TERNARY-COMPLEX MECHANISM

Peter Ruoff
Department of Chemistry, Rogaland University Center, Ullandhaug, 4004 Stavanger, Norway

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There is presently no clearly established mechanism for the reduction of nitrate by NADH catalyzed by NADH: nitrate reductase (EC 1.6.6.1). We found recently the first example of NADH substrate inhibition for higher plant nitrate reductase. In this Communication we show that the NADH substrate inhibition supports a ternary-complex mechanism. Our results are not consistent with a substituted-enzyme (ping-pong) mechanism.

Nitrate reductase (NR, EC 1.6.6.1-3) catalyzes the reduction of nitrate to nitrite by reduced pyridine nucleotides:

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

Process R1 is the first and rate-determining step in the transformation of nitrate to organically-bound nitrogen, a process found in many unicellular organisms, most fungi, and all higher plants (1-3). Only a few kinetic studies have so far been performed with nitrate reductase and no unique mechanism has been established. While some studies indicate that process R1 proceeds via a substituted-enzyme (ping-pong) mechanism (4), other workers conclude that the mechanism involves a ternary enzyme substrate complex (5). Our interest in nitrate reductase originated from the light-induced oscillatory variation of its activity which has been observed in leaves of several higher plants (6-8). We have recently found that NADH: nitrate reductase shows NADH substrate inhibition, which opens the

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possibility of bistability and oscillatory behavior when reaction R1 is far from equilibrium (9).

We have now studied the NADH substrate inhibition at different nitrate levels and report here that inhibition by NADH for corn leaf nitrate reductase provides evidence for a ternary-complex mechanism but not for a ping-pong mechanism. The results are further in agreement with a suggestion by Howard and Solomonson (5) that earlier (4) found parallel line patterns in double-reciprocal plots not necessarily are a sufficient condition for a ping-pong mechanism, but may be due to solvent effects where the rapid equilibrium assumption does not hold.

MATERIALS AND METHOD

Corn leaf nitrate reductase was purified as described earlier (9). Specific activities of the purified enzyme were about 80-90 units/mg, which are comparable to the specific activities obtained by Howard and Solomonson (5) for the *Chlorella vulgaris* nitrate reductase. Kinetic experiments were performed at 30°C in 0.1 M Tris-HCl buffer (pH 7.5, 1 mM EDTA) by following spectrophotometrically at 340 nm (9) the amount of NADH consumed. All **NADH solutions used in this study were prepared fresh and used promptly.**

RESULTS AND DISCUSSION

In a bi bi ternary-complex mechanism both substrates (here NADH and nitrate) must first bind to the enzyme before products can be released, while in ping-pong mechanisms a product has to be released before the other substrate can bind to the enzyme (10,11).

In ping-pong and multisite ping-pong mechanisms one expects (and sees) competitive substrate inhibition, i.e., the substrate inhibition is largest when the concentration of *noninhibitory* substrate A (in our case nitrate) is low. The substrate inhibition disappears when the noninhibitory substrate is increased (10,11).

Ternary-complex mechanisms, on the other hand, can exhibit uncompetitive substrate inhibition. In such cases the substrate inhibition is greatest at high concentrations of the noninhibitory

substrate A, while at low levels of A no substrate inhibition at any concentration of the inhibitory substrate B (here NADH) may be detected (10,11). An interesting discussion of substrate inhibition in terms of free energy profiles is given by Noyes (12).

Figure 1 shows initial reaction rates of process R1 as a function of initial NADH concentration at different nitrate levels for NADH: corn leaf nitrate reductase. The NADH substrate inhibition is more pronounced at increasing levels of nitrate (the noninhibitory cosubstrate) and finally reaches an asymptotic curve at high nitrate concentrations (>25 mM). We are not aware of any ping-pong mechanism where an increase in the noninhibitory substrate intensifies the effect of the inhibitory substrate.

Solid lines in Figure 1 represent a fit of equation 1 to the experimental data points.

$$v_0 = \frac{V_{max} \cdot a \cdot b}{k_{ia} \cdot k_{mb} + k_{ma} \cdot b + k_{mb} \cdot a + ab\left(1 + \frac{b}{k_{si}}\right)} \qquad (1)$$

Equation 1 describes the initial rate v_0 as a function of nitrate concentration "a" and NADH concentration "b" for NADH substrate inhibition of a ternary-complex mechanism (11), independent whether the mechanism is of compulsory-order or has random binding of the substrates (Figure 2). Table I lists the optimized parameters which are within the range of earlier published values (1), and which provide a good fit to the experimental data points even if the nitrate ion concentration "a" differs by three orders of magnitude. The sensitivity of v_0 (equation 1) to the parameters of Table I can be expressed as $\partial v_0/\partial X$, i.e., as the partial derivative of v_0 with respect to the parameter X. These "sensitivity coefficients" are given in Table II using an initial NADH concentration of 100 µM and nitrate ion concentrations at 25 µM, 250 µM, 2500 µM, and 25 mM. From Table II we see that v_0 is most sensitive upon variations in k_{mb}.

While an earlier study of corn leaf nitrate reductase found parallel line patterns in double-reciprocal plots and concluded in favor of a ping-pong mechanism (4), Howard and Solomonsen (5) have pointed out that the parallel line patterns may be the result of that particular

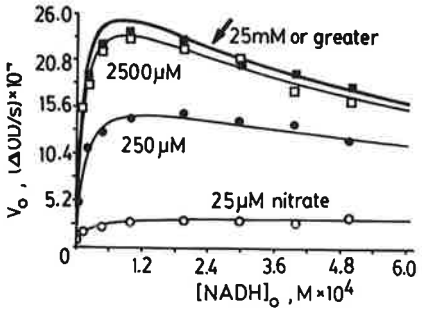

Figure 1. Initial rate v_0 measured as optical density changes per second, ΔOD/s, at 340 nm as a function of initial NADH concentration and different constant nitrate levels. Points indicate experimental values, while solid lines show v_0 calculated from equation 1 for parameter values listed in Table I with nitrate concentrations as indicated in the figure. Nitrate concentrations for experimental points: open circles, 25 μM; solid circles, 250 μM; open squares, 2500 μM; solid squares, 25 mM. The arrow indicates the asymptotic curve which is reached when nitrate concentrations are 25 mM or higher. Experimental data points are the mean of two measurements with an observed uncertainty between 6-13%.

Table I. Optimized Parameter Values

Parameter	Value
k_{ia}	3×10^{-4} M
k_{ma}	2×10^{-4} M
k_{mb}	1.2×10^{-5} M
k_{si}	6×10^{-4} M
v_{max}	3.2×10^{-3} ΔOD/s

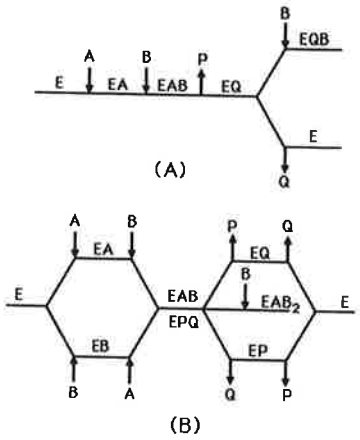

Figure 2. Ternary-order mechanisms (notation is due to Cleland (13)) leading to an initial rate law described by equation 1. (A) Compulsory-order mechanism (steady state assumption; for derivation see for example reference 11). (B) Random bi bi mechanism (rapid equilibrium assumption). For derivation see reference 14. Note that in the notation of reference 14 k_{ma} and k_{mb} now become equilibrium constants k_a and k_b, respectively; k_{is} is the equilibrium constant for the formed dead-end product EAB_2, defined by $k_{is} = ([EAB][B])/[EAB_2]$, with A = NO_3^-, B = NADH, P = NAD^+, Q = NO_2^-, and E = nitrate reductase.

Table II. $\partial v_0/\partial X$ (in s^{-1}) for Different Initial Nitrate Ion Concentrations[a]

X	$(\frac{\partial v_0}{\partial X})$ 25μM	$(\frac{\partial v_0}{\partial X})$ 250μM	$(\frac{\partial v_0}{\partial X})$ 2500μM	$(\frac{\partial v_0}{\partial X})$ 25mM
v_{max}	0.09323	0.44830	0.72408	0.77154
k_{ia}	-0.13349	-0.30869	-0.08053	-0.00914
k_{ma}	-1.11245	-2.57241	-0.67109	-0.07620
k_{mb}	-3.61546	-14.14827	-18.79052	-19.27742
k_{si}	0.00773	0.17864	0.46603	0.52913

[a] $[NADH]_0 = 100\mu M$

reaction medium. This reaction medium was different from that used in the present study.

The observation of substrate inhibition will make it more secure to assign (or reject) a ping-pong mechanism to an observed parallel initial velocity pattern (10). Further work is being considered to test kinetic mechanisms by NAD(P)H substrate inhibition for different nitrate reductase preparations.

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