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Activation of Nitrite Reductase from *Escherichia coli* K12 by Oxidized Nicotinamide–Adenine Dinucleotide

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Nitrite reductase from *Escherichia coli* K12 requires the presence of NAD^+, one of the products of the reduction of NO_2^- by NADH, for full activity. The effect is observed with both crude extracts and purified enzyme. NAD^+ also acts as a product inhibitor at high concentrations, and plots of initial rate against NAD^+ concentration are bell-shaped. The maximum occurs at about 1 mM-NAD^+, but increases with increasing NADH concentration. In the presence of 1 mM-NAD^+ and saturating NO_2^- (2 mM) the Michaelis constant for NADH is about 16 μM. The Michaelis constant for NO_2^- is about 5 μM and is largely independent of the NAD^+ concentration. Similar but more pronounced effects of NAD^+ are observed with hydroxylamine as electron acceptor instead of NO_2^-. The maximum rate of NADH oxidation by hydroxylamine is about 5.4 times greater than the maximum rate of NADH oxidation by NO_2^- when assayed with the same volume of the same preparation of purified enzyme. The Michaelis constant for hydroxylamine is 5.3 mM, however, about 1000 times higher than for NO_2^-. These results are consistent with a mechanism in which the same enzyme–hydroxylamine complex occurs as an intermediate in both reactions.

The activity of NADH-dependent nitrite reductase (NADH–nitrite oxidoreductase, EC 1.6.6.4) in cell-free extracts of *Escherichia coli* K12 is sensitive to NAD^+, one of the products of the reaction (Cornish-Bowden *et al.*, 1973). When the rate of oxidation of NADH by NO_2^- and dissolved O_2 is recorded in open cuvettes in the absence of added NAD^+, the reaction is initially slow, but accelerates markedly during the first few minutes after mixing. This acceleration also occurs if the enzyme is preincubated with either NADH or NO_2^-, but it is greatly decreased by preincubation with 0.1 mM-NAD^+ and is undetectable when untreated enzyme is added to a reaction mixture containing 10 μM-NAD^+.

These preliminary observations suggested that nitrite reductase is activated by its product NAD^+. Although this is an unusual phenomenon, it is not unprecedented, as lipoamide dehydrogenase behaves similarly (Massey & Veeger, 1961; Massey, 1963). We now report experiments designed to delineate the effects of NAD^+ and NADH on nitrite reductase activity, using better characterized enzyme (see Coleman *et al.*, 1978) than was available in our preliminary studies.

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Materials and Methods

Materials were as given in the preceding paper (Coleman *et al.*, 1978) and nitrite reductase was purified from *E. coli* K12 as described there. The first of the two assays described there was used for kinetic experiments with appropriate and obvious modifications to allow the effects of variations in substrate and NAD^+ concentrations to be monitored. The parameters V and K_m of the Michaelis–Menten equation were estimated by the graphical method of Eisenthal & Cornish-Bowden (1974), but the final values given in the present paper were calculated by means of the computer program described by Cornish-Bowden & Eisenthal (1974).

NAD^+ glycohydrolase (EC 3.2.2.5) was extracted from *Neurospora crassa* mycelia and partially purified in this laboratory by Miss B. E. Pirog by the procedure of Kaplan (1955). The stock solution contained about 0.17 μkat of NAD^+ glycohydrolase/ml.

Results

Activation of nitrite reductase by NAD^+

Table 1 shows the results of assaying nitrite reductase in the presence and absence of NAD^+

Table 1. *Effect of NAD⁺ on nitrite reductase*
Experiments were done in the presence of 0.25mM-NADH at pH8.0 and 30°C. 'NADase' (NAD⁺ glycohydrolase) catalyses the irreversible hydrolysis of NAD⁺ but has no effect on NADH. Rates are shown as percentages of the rate observed with 2mM-$NaNO_2$ and no NAD⁺.

Addition to reaction mixture	Relative rate
2mM-$NaNO_2$	100
1mM-NAD⁺	18
1mM-NAD⁺ + NADase (17nkat/ml)	26
1mM-NAD⁺ + 2mM-$NaNO_2$	230
1mM-NAD⁺ + 2mM-$NaNO_2$ + NADase (17nkat/ml)	26

glycohydrolase, an enzyme that catalyses the irreversible hydrolysis of NAD⁺ but has no effect on NADH. When NAD⁺ glycohydrolase was not present, there was some reaction in the absence of added NAD⁺, but this was increased more than 2-fold by the addition of NAD⁺. However, the bulk of the activity in the absence of NAD⁺ can be ascribed to the effects of NAD⁺, which was probably present as an impurity, because it was largely eliminated by the addition of NAD⁺ glycohydrolase, and when this enzyme was present, addition of NAD⁺ produced no lasting activation of nitrite reductase. These results are similar to those obtained by Massey & Veeger (1961) and Massey (1963) in studies of the NAD⁺ activation of lipoamide dehydrogenase, and to those obtained with crude extracts of nitrite reductase (Cornish-Bowden *et al.*, 1973).

The capacity of nitrite reductase to be activated by NAD⁺ increased as it was purified. In crude extracts and partially purified samples the ratio of activities in the presence and in the absence of 1mM-NAD⁺ was about 2.5, but after the last stage in the purification procedure (Coleman *et al.*, 1978) it was 5.0.

There was no activation of nitrite reductase when 1mM-NAD⁺ was replaced in the enzyme assay by 1mM-ADP or 1mM-NADP⁺, and rates were similar to those observed in the absence of added NAD⁺. Activation by NAD⁺ also occurred in the absence of ascorbate and dithiothreitol and was not therefore an artifact of the presence of either of these species.

Product inhibition by NAD⁺

At high concentrations, NAD⁺ acted as a product inhibitor of nitrite reductase. At concentrations of NADH up to 0.1mM, plots of reaction rate against NAD⁺ were clearly defined bell-shaped curves with maxima at NAD⁺ concentrations that increased with the NADH concentration (Fig. 1). At higher NADH concentrations the behaviour was qualitatively similar to that observed at lower concentrations, but

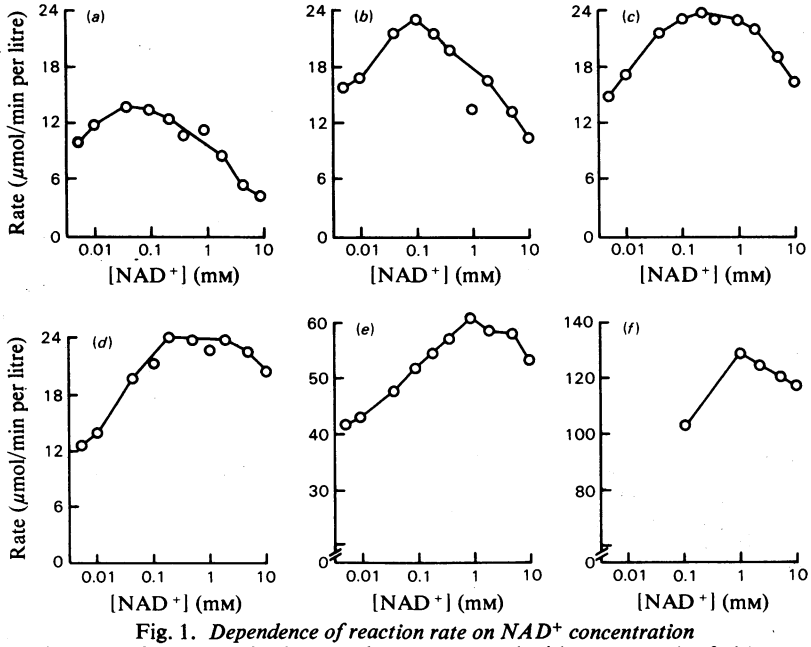


Fig. 1. *Dependence of reaction rate on NAD⁺ concentration*
Experiments were done at 30°C and pH8.0 in a total volume of 1ml with path-length of either 1cm (*a–d*) or 2mm (*e* and *f*). The concentration of NO_2^- was 2mM in every case. Concentration of NADH were as follows: (*a*) 0.02mM, (*b*) 0.05mM, (*c*) 0.1mM, (*d*) 0.25mM, (*e*) 0.5mM, (*f*) 1.0mM.

the data were less precise because of the need to use 2mm-light-path cuvettes and much higher enzyme concentrations in these experiments.

Absence of substrate inhibition by NADH

Although preliminary experiments with crude extracts of nitrite reductase suggested that it was inhibited by NADH at high concentrations (Cornish-Bowden *et al.*, 1973), this property was not detected in subsequent work with the purified enzyme, even at NADH concentrations as high as measurements could be made in 2mm-light-path cuvettes. Instead the reaction is described to within the limits of experimental error by the Michaelis–Menten equation. Thus it is possible to characterize the effects of NAD^+ in terms of its effects on the Michaelis–Menten parameters for NADH.

Effect on NAD^+ on apparent Michaelis–Menten parameters for NADH

Fig. 2 shows the variation with the NAD^+ concentration of $K_m^{app.}$ and $V^{app.}$, the apparent values of K_m and V respectively for NADH as substrate. It is clear that $K_m^{app.}$ increased with [NAD^+], but the scatter of points makes it uncertain whether there was any effect on $V^{app.}$. The dependence of $K^{app.}$ on [NAD^+] was apparently non-linear, and the points gave a significantly better fit to a rectangular hyperbola with a positive intercept on the ordinate than to a straight line, with a value of $F = 10.6$ in an F test of the significance of the effect of introducing the third parameter. This is significant at the 95% level of confidence ($F = 4.49$ for a 95% test with 16 and 1 degrees of freedom for the smaller and larger mean squares respectively), but in view of the large scatter and the absence of knowledge of the validity of the assumptions implicit in such a test we do not believe that it would be appropriate to draw any mechanistic conclusions from the non-linearity until more precise data are available.

The large scatter of $V^{app.}$ values in Fig. 2 was caused primarily by day-to-day variations in the enzyme preparation. In efforts to establish whether $V^{app.}$ does vary with [NAD^+] we took advantage of the clearer trend in $K_m^{app.}$ values by using the fact that the rate must be $0.5\,V^{app.}$ when [NADH] = $K_m^{app.}$, for any NAD^+ concentration. Results of measurements at three NAD^+ concentrations are shown in Table 2, from which it is clear that $V^{app.}$ does not vary significantly with [NAD^+]. It appears therefore that NAD^+ is a competitive inhibitor of nitrite reductase, though it is unclear whether the inhibition is linear.

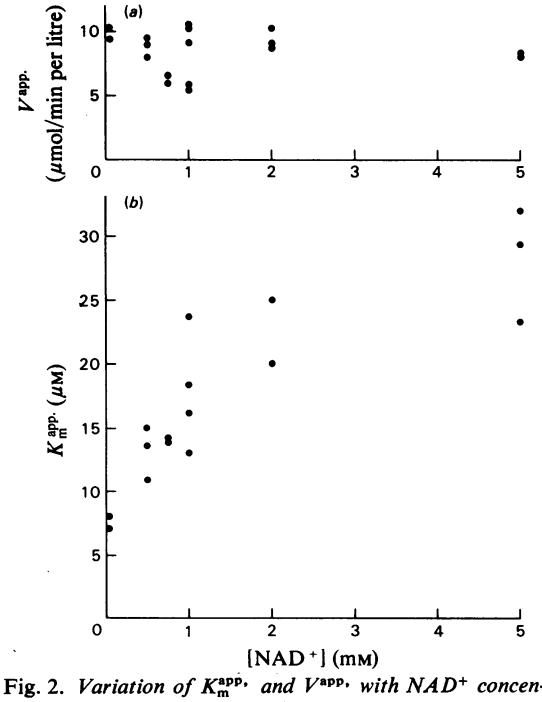


Fig. 2. *Variation of $K_m^{app.}$ and $V^{app.}$ with NAD^+ concentration*

At each NAD^+ concentration, $K_m^{app.}$ and $V^{app.}$ were determined by varying the concentration of NADH with constant 2mM-$NaNO_2$. The same amount of enzyme was used in each case. (*a*) $V^{app.}$; (*b*) $K_m^{app.}$.

Dependence of rate on NO_2^- concentration

The Michaelis constant for NO_2^- was determined by varying the NO_2^- concentration in the range 0–24μM at constant concentrations of NADH (0.25mM) and NAD^+ (1 or 10mM). Values of $K_m^{app.}$ in the range 4.7–5.0μM were obtained with 1mM-NAD^+ and 5.5μM with 10mM-NAD^+. Thus any effect of NAD^+ on the NO_2^--dependence is slight.

Table 2. *Effect of NAD^+ on V*

This experiment was designed to overcome the difficulty evident in Fig. 2(*a*) of measuring V reproducibly. The NADH concentrations were estimated from Fig. 2(*b*) as those that would give half-maximal velocity at the NAD^+ concentrations used, i.e. in each experiment the NADH concentration was equal to $K_m^{app.}$. Expts. A and B were carried out independently (on different days) using enzyme from a single preparation. Values of v are means ± S.E.M. for the numbers of preparations in parentheses.

	[NADH] (μM)	[NAD^+] (mM)	v (μmol. $min^{-1} \cdot l^{-1}$)
Expt. A	7.5	0.06	3.90 ± 0.054 (7)
	12.0	0.5	3.90 ± 0.067 (7)
Expt. B	7.5	0.06	3.00 ± 0.085 (7)
	17.0	1.0	3.20 ± 0.177 (7)

NO_2^- cannot be replaced as electron acceptor by sulphite. In the presence of sulphite (2–20mM) but no NO_2^-, the rate of oxidation of NADH was similar to the blank rate in the absence of any acceptor. Thus nitrite reductase from *E. coli* K12 cannot act as a sulphite reductase. Moreover, the $K_m^{app.}$ for NO_2^- increased only slightly from 5 to 6μM, in the presence of 100μM-sulphite. Thus sulphite is a much weaker inhibitor of this enzyme than of nitrite reductase from *E. coli* B_n for which Kemp & Atkinson (1966) reported a competitive inhibition constant of 30μM.

Hydroxylamine as electron acceptor

Nitrite reductase from *E. coli* K12 also acts as a hydroxylamine reductase with kinetics that are qualitatively similar to those observed with NO_2^- as substrate. There are, however, substantial numerical differences: the purified enzyme had $K_m^{app.} = 5.3$mM for hydroxylamine in the presence of 0.25mM-NADH and 1mM-NAD^+, 1000 times higher than the value of 5μM observed for NO_2^-; the $V^{app.}$ for hydroxylamine was 5.4 times higher than for NO_2^-, when assayed with the same volume of the same preparation of purified enzyme. As Kemp & Atkinson (1966) remarked in the context of *E. coli* B_n, such results eliminate free hydroxylamine as a possible intermediate in the reduction of NO_2^- to ammonia, though they permit the occurrence of enzyme-bound hydroxylamine as an intermediate.

The enzyme is again activated by NAD^+ when hydroxylamine is reduced, but the amount of activation is much greater than with NO_2^- (Fig. 3). With 30mM-hydroxylamine and 0.25mM-NADH, the maximum rate occurs with 1mM-NAD^+, in the same range of concentrations as required for maximum activation in the reduction of NO_2^-.

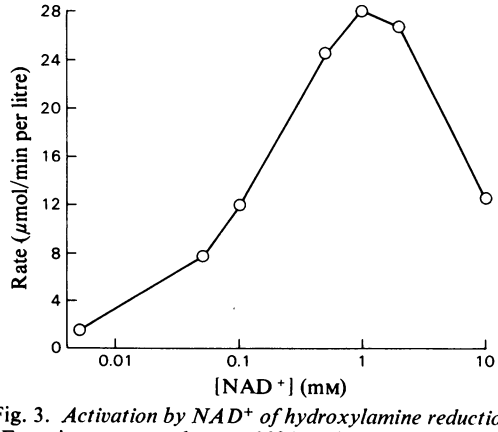


Fig. 3. *Activation by NAD^+ of hydroxylamine reduction*
Experiments were done at 30°C and pH8.0 in a total volume of 1ml containing 0.25mM-NADH and 30mM-hydroxylamine.

Table 3. *Competition between NO_2^- and hydroxylamine*
Experiments were done at 30°C and pH8.0 in the presence of 0.25mM-NADH. Rates are expressed as percentages of the rate (38.5μmol/min per litre) observed with 1mM-NAD^+ and 2mM-NO_2^-. The same volume of enzyme from a single preparation was used in each case.

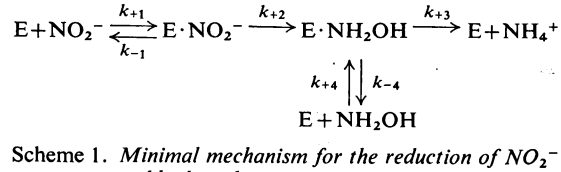
Additions to cuvette	Relative rate
1mM-NAD^+ + 2mM-$NaNO_2$	100
2mM-$NaNO_2$	21
1mM-NAD^+ + 10mM-hydroxylamine	210
10mM-hydroxylamine	29
1mM-NAD^+ + 2mM-$NaNO_2$ + 10mM-hydroxylamine	120
2mM-$NaNO_2$ + 10mM-hydroxylamine	17

NO_2^- and hydroxylamine appear to react at the same site on the enzyme, as they show competitive kinetics with respect to one another. Table 3 shows the rates observed with various combinations of NO_2^-, hydroxylamine and NAD^+. In the presence of 1mM-NAD^+, the rate with both 2mM-NO_2^- and 10mM-hydroxylamine present is much less than with 10mM-hydroxylamine alone, and only slightly higher than with 2mM-NO_2^- alone. These results are similar to those observed by Cresswell *et al.* (1965) with leaf extracts from *Cucurbita pepo* L (vegetable marrow). They found that hydroxylamine reduction was strongly inhibited by NO_2^-, but that NO_2^- reduction was negligibly affected by hydroxylamine.

Discussion

The results given in the present paper are consistent with the view that enzyme-bound hydroxylamine is an intermediate in the six-electron reduction of NO_2^- to ammonia by three molecules of NADH, and that reduction of hydroxylamine proceeds via the same intermediate. Although hydroxylamine is a much poorer substrate than NO_2^- for nitrite reductase, with a $V^{app.}/K_m^{app.}$ about 0.5% that of NO_2^-, the maximum velocity is about 5 times greater for hydroxylamine.

A minimal kinetic scheme for discussing the NO_2^- and hydroxylamine kinetics is shown in Scheme 1. This makes no attempt to incorporate the involvement of NADH and NAD^+, or to define the nature of



Scheme 1. *Minimal mechanism for the reduction of NO_2^- and hydroxylamine to ammonia*

the four-electron reduction of enzyme-bound NO_2^- to enzyme-bound hydroxylamine. Thus the kinetic constants are apparent values that apply with 0.25 mM-NADH and 1 mM-NAD^+. With these kinetic constants as defined by Scheme 1, the rate (v) of NADH oxidation is given by the following equation:

$$\frac{v}{e_0} = \frac{k_{+1}k_{+2}(3k_{+3}+2k_{-4})[NO_2^-]+(k_{-1}+k_{+2})k_{+3}k_{+4}[NH_2OH]}{(k_{-1}+k_{+2})(k_{+3}+k_{-4})+k_{+1}(k_{+2}+k_{+3}+k_{-4})[NO_2^-]+(k_{-1}+k_{+2})k_{+4}[NH_2OH]}$$

(The factors 3 and 2 occur in this equation because each mol of NO_2^- yields 3 mol of NAD^+ on complete reduction to ammonia but only 2 mol of NAD^+ on reduction to hydroxylamine). The stoicheiometric conversion of NO_2^- into ammonia (see Coleman *et al.*, 1978) indicates that k_{-4} is small in comparison with k_{+3}, and so the equation may be simplified as follows:

$$\frac{v}{e_0} = \frac{3k_{+1}k_{+2}k_{+3}[NO_2^-]+(k_{-1}+k_{+2})k_{+3}k_{+4}[NH_2OH]}{(k_{-1}+k_{+2})k_{+3}+k_{+1}(k_{+2}+k_{+3})[NO_2^-]+(k_{-1}+k_{+2})k_{+4}[NH_2OH]}$$

Then the apparent Michaelis–Menten parameters for NO_2^- in the absence of hydroxylamine are $K_m^{app.} = (k_{-1}+k_{+2})k_{+3}/k_{+1}(k_{+2}+k_{+3})$ and $V^{app.} = 3k_{+2}k_{+3}e_0/(k_{+2}+k_{+3})$; for hydroxylamine in the absence of NO_2^-, $K_m^{app.} = k_{+3}/k_{+4}$ and $V^{app.} = k_{+3}e_0$. These are consistent with the observation that $V^{app.}$ is 5.4 times greater for hydroxylamine than for NO_2^- if $k_{+3} = 15k_{+2}$, and with the observation that $K_m^{app.}$ is 1000 times greater for hydroxylamine than for NO_2^- if k_{+4} does not exceed $0.016k_{+1}$. These results apply to the enzyme activated by 1 mM-NAD^+ and they shed some light on the nature of the activation. The fact that the reduction of hydroxylamine is activated by NAD^+ indicates that the effective value of k_{+3} varies with [NAD^+], and the simplest hypothesis consistent with the data is that NAD^+ activation derives solely from this dependence. This accounts for the much more pronounced activation of the hydroxylamine reaction compared with the NO_2^- reaction (Figs. 1 and 3), because any change in k_{+3} must affect the rate of reduction of hydroxylamine directly whereas the rate of reduction of NO_2^- is appreciably affected by variation in k_{+3} only when k_{+3} is approximately equal to or smaller than k_{+2}.

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