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The steady-state kinetics of the NADH-dependent nitrite reductase from *Escherichia coli* K12

Nitrite and hydroxylamine reduction

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The reduction of both NO_2^- and hydroxylamine by the NADH-dependent nitrite reductase of *Escherichia coli* K12 (EC 1.6.6.4) appears to follow Michaelis–Menten kinetics over a wide range of NADH concentrations. Substrate inhibition can, however, be detected at low concentrations of the product NAD^+. In addition, NAD^+ displays mixed product inhibition with respect to NADH and mixed or uncompetitive inhibition with respect to hydroxylamine. These inhibition characteristics are consistent with a mechanism in which hydroxylamine binds during catalysis to a different enzyme form from that generated when NAD^+ is released. The apparent maximum velocity with NADH as varied substrate increases as the NAD^+ concentration increases from 0.05 to 0.7mM with 1mM-NO_2^- or 100mM-hydroxylamine as oxidized substrate. This increase is more marked for hydroxylamine reduction than for NO_2^- reduction. Models incorporating only one binding site for NAD can account for the variation in the Michaelis–Menten parameters for both NADH and hydroxylamine with [NAD^+] for hydroxylamine reduction. According to these models, activation of the reaction occurs by reversal of an over-reduction of the enzyme by NADH. If the observed activation of the enzyme by NAD^+ derives both from activation of the generation of the enzyme–hydroxylamine complex from the enzyme–NO_2^- complex during NO_2^- reduction and from activation of the reduction of the enzyme–hydroxylamine complex to form NH_4^+, then the variation of $V^{app.}$ for NO_2^- or hydroxylamine with [NAD^+] is consistent with the occurrence of the same enzyme–hydroxylamine complex as an intermediate in both reactions.

The NADH-dependent nitrite reductase from *Escherichia coli* K12 (NADH:nitrite oxidoreductase, EC 1.6.6.4) is unusual because it shows full activity only in the presence of NAD^+, one of the products of the reaction: no nitrite-dependent NADH oxidation could be detected when the enzyme was assayed in the presence of NAD^+ glycohydrolase (EC 3.2.2.5) (Coleman *et al.*, 1978). NAD^+ is also a product inhibitor.

In addition to the six-electron reduction of NO_2^- to NH_4^+, the enzyme catalyses the reduction of hydroxylamine and single-electron acceptors such as cytochrome *c*. These reactions are also NADH-dependent. The maximum rate of NADH oxidation by hydroxylamine (with 0.25mM-NADH and 1mM-NAD^+) is about 5.4 times greater than the maximum rate of NADH oxidation by NO_2^-. These observations are consistent with the occurrence of the same enzyme–hydroxylamine complex as an intermediate in the reduction of both nitrite and hydroxylamine (Coleman *et al.*, 1978).

In the present work we have studied the reduction of hydroxylamine in detail, because this two-electron reduction is potentially more amenable to analysis than the full reaction, as well as being more markedly sensitive to NAD^+. The availability of more highly purified enzyme than before (Coleman *et al.*, 1978) has permitted more informative kinetic studies to be carried out.

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Methods

Enzymes

Nitrite reductase was purified from *E. coli* strain OR75Ch15 as described by Jackson *et al.* (1981), and appeared to be more than 95% pure as judged by sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

NAD^+ glycohydrolase (EC 3.2.2.5) was extracted from *Neurospora crassa* mycelia and partially purified by the procedure of Kaplan (1955). The amount of NAD^+ hydrolysed during an 8-min incubation was assessed by the decrease in amount of NADH formed from NAD^+ during reduction by ethanol, catalysed by yeast alcohol dehydrogenase, estimated as below from A_{340} measurements.

Measurement of initial rates of NADH-dependent reduction of NO_2^- and hydroxylamine

Open sample and reference cuvettes contained various concentrations of NAD^+, NADH and NO_2^- or hydroxylamine in TEA (50mM-Tris/HCl/5mM-EDTA/5mM-ascorbate) buffer, pH8.0 at 30°C. The assay volume was 1ml in 1cm light path cuvettes, for NADH up to 200μM, but 0.5ml in 0.5cm light path cuvettes at higher concentrations of NADH. The reaction was initiated by adding enzyme to the sample cuvette. The rate of decrease in A_{340} in a Cary 14 recording spectrophotometer (Applied Physics Corp., Monrovia, CA, U.S.A.) was measured. Initial rates were calculated from orthogonal polynomials fitted to the progress curves. The rate of NADH oxidation in the absence of NO_2^- or hydroxylamine was measured and subtracted to give the nitrite or hydroxylamine reductase activity.

Preparation of enzyme for the determination of kinetic constants

Nitrite reductase from the DEAE–Sephadex pool (i.e. the final step of the purification) was precipitated with 50%-satd. $(NH_4)_2SO_4$, redissolved and desalted by passage through a Sephadex G-25 column equilibrated with TEA or TE (i.e. TEA with ascorbate omitted) buffer containing 50% (w/v) glycerol, supplemented with 0.01mM-FAD and 1mM-NO_2^- or 50mM-hydroxylamine if appropriate ('stabilizing buffer'). The enzyme was stored unfrozen at −20°C until just before use. The enzyme was diluted with stabilizing buffer and each series of rate determinations was completed within 1h.

Determination of kinetic constants

The Michaelis–Menten parameters V and K_m were estimated from the direct linear plot of Eisenthal & Cornish-Bowden (1974) as modified by Cornish-Bowden & Eisenthal (1978). A computer program was used to calculate the results (Cornish-Bowden *et al.*, 1978).

Derivation of rate equations

In the more complex cases equations were derived by the method of King & Altman (1956) using a computer program. The expected number of terms for each species were checked by the method of Chou *et al.* (1979). The numerator of the rate equation was derived by a method based on the structural rule of Wong & Hanes (1962). The computer program used for all of these calculations is considerably extended beyond that described previously (Cornish-Bowden, 1977), and is available from A. C.-B. on request.

Results

Substrate inhibition of nitrite reductase by NADH

Plots of rate of reaction against NADH concentration with low [NAD^+] and 1mM-NO_2^- deviated from the pattern predicted by the Michaelis–Menten equation as [NADH] increased towards 0.4mM (Fig. 1). With 0.02mM- and 0.25mM-NAD^+ there was a plateau, followed by a decline in rate as [NADH] increased, the plateau occurring at lower [NADH] with 0.02mM-NAD^+. This decline in rate was not caused by an artefact of spectrophotometer sensitivity as the same rate was obtained with 200μM-NADH when the enzyme was assayed in 1cm or 0.5cm light path cuvettes. No deviation from Michaelis–Menten kinetics could be detected in the range of [NADH] up to 0.4mM at 1mM- and 5mM-NAD^+. A similar decline in velocity with increasing [NADH] was noted at 0.05mM-NAD^+ with 100mM-hydroxylamine as oxidized substrate. Hence, in spite of an earlier report (Coleman *et al.*, 1978), NADH is a substrate inhibitor of nitrite and hydroxylamine reduction.

Effect of NAD^+ glycohydrolase on hydroxylamine and nitrite reduction

Coleman *et al.* (1978) observed that at high [NADH] no nitrite-dependent NADH oxidation could be detected in the presence of NAD^+ glycohydrolase, an enzyme that catalyses the irreversible hydrolysis of NAD^+ but has no effect on NADH. Similarly, at high [NADH] no hydroxylamine-dependent NADH oxidation could be detected in the presence of NAD^+ glycohydrolase (Table 1). The low rate observed in the absence of NAD^+ glycohydrolase and added NAD^+ can possibly be ascribed to NAD^+ present as an impurity in preparations of NADH. At 0.01mM-NADH, however, an increased rate of NADH oxidation was observed in the presence of 1mM-NO_2^- and NAD^+ glycohydrolase. This suggests it is not necessary for NAD^+ to be bound to the enzyme for catalysis to occur. Presumably, 0.01mM-NADH is insufficient either to convert the enzyme completely to an

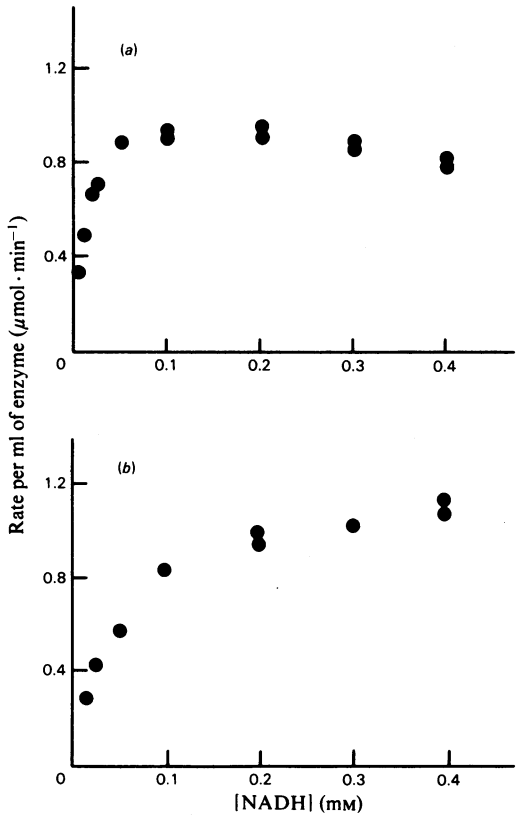


Fig. 1. *Substrate inhibition of nitrite reduction by NADH*
The rate of nitrite-dependent NADH oxidation was measured over the range 0.005–0.4 mM-nitrite using 0.5 cm light path cuvettes with assay mixtures, volume 0.5 ml, containing 1 mM-NO_2^- and NAD^+ at (*a*) 0.02 mM or (*b*) 5 mM. Reaction velocity is expressed as μmol of NADH oxidized/min per ml of enzyme. The behaviour with 0.025- and 1 mM-NAD^+ (not shown) was intermediate between the cases shown.

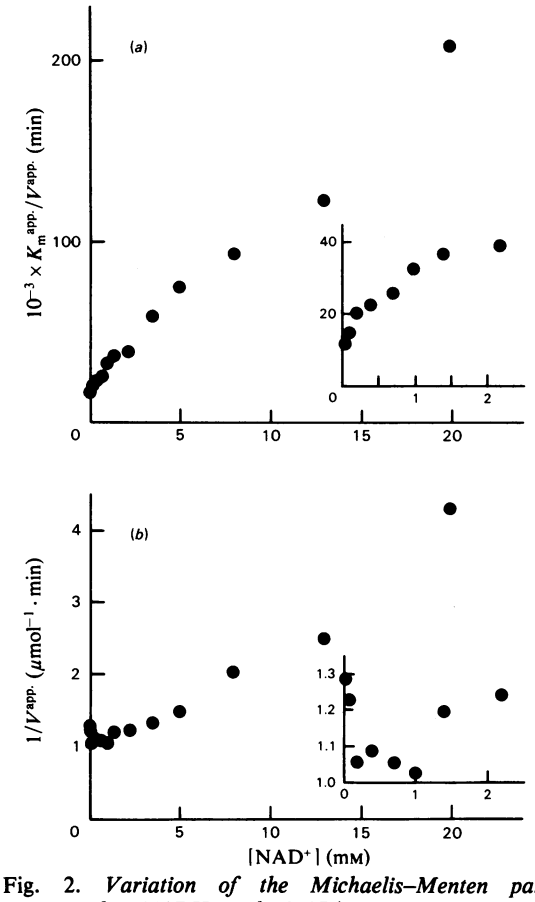


Fig. 2. *Variation of the Michaelis–Menten parameters for NADH with NAD^+ concentration with hydroxylamine as oxidized substrate*
[NADH] was varied in the range 0.02–0.1 mM for [NAD^+] below 0.4 mM and in the range 0.004–0.2 mM- for [NAD^+] 0.4 mM and above. Identical quantities of the same enzyme preparation were used in each determination. The hydroxylamine concentration was 101 mM.

Table 1. *Activation of nitrite reductase by NAD^+*
Rates are shown as percentages of the rate observed with 0.2 mM-NADH, 100 mM-hydroxylamine and 0.4 mM-NAD^+, and were measured in duplicate (apart from three single determinations marked *) with nitrite reductase of specific activity 1.12 kat · kg^{-1}. 'NADase' (NAD^+ glycohydrolase) catalyses the irreversible hydrolysis of NAD^+ but has no effect on NADH; when present its activity in the reaction mixture was 13.5 nkat · ml^{-1}.

Reaction mixture	Relative rate
0.2 mM-NADH, 1 mM-$NaNO_2$	14*
0.2 mM-NADH, 0.4 mM-NAD^+	4.7*
0.2 mM-NADH, 0.4 mM-NAD^+, NADase	10
0.2 mM-NADH, NADase	10
0.2 mM-NADH, 0.4 mM-NAD^+, 1 mM-$NaNO_2$	39
0.2 mM-NADH, 0.4 mM-NAD^+, 1 mM-$NaNO_2$, NADase	10
0.2 mM-NADH, 1 mM-$NaNO_2$, NADase	10
0.2 mM-NADH, 100 mM-NH_2OH, NADase	10*
0.2 mM-NADH, 100 mM-NH_2OH, 0.4 mM-NAD^+	100
0.01 mM-NADH, NADase	9.2
0.01 mM-NADH, 1 mM-$NaNO_2$	12
0.01 mM-NADH, 1 mM-$NaNO_2$, NADase	12

inactive form by 'over-reducing' it (see the Discussion) or to saturate an inhibitory site. Cornish-Bowden *et al.* (1973) found that the rate of O_2 reduction increased when NO_2^- was added to assay mixtures and the possibility that the increased rate with 10μM-NADH when 1mM-NO_2^- was present was due to O_2 reduction has not been excluded. However, with 0.2mM-NADH the rate of NADH oxidation did not increase when NO_2^- was added, suggesting that there was no increase in the rate of O_2 reduction.

Effect of NAD$^+$ on the reduction of hydroxylamine

Fig. 2 shows the variation with NAD$^+$ concentration of the apparent Michaelis–Menten parameters for NADH as varied substrate in the presence of 100mM-hydroxylamine. Over the range of [NADH] tested there was no significant deviation from Michaelis–Menten kinetics, slopes of Hill plots of $\log[v/(V^{app.}-v)]$ against log[NADH] being in the range 0.96–1.03. The apparent value of K_m/V increased with [NAD$^+$] over the whole range considered, with a slight departure from linearity. The apparent value of $1/V$ clearly increased with [NAD$^+$] at concentrations above 2mM, but appeared to pass through a minimum at about 1mM-NAD$^+$. Thus NAD$^+$ acts as a mixed inhibitor with respect to NADH, in addition to its effect as an activator at low concentrations.

The variation of the apparent Michaelis–Menten parameters for hydroxylamine with [NAD$^+$] was studied in the presence of 0.25mM-NADH (Fig. 3).

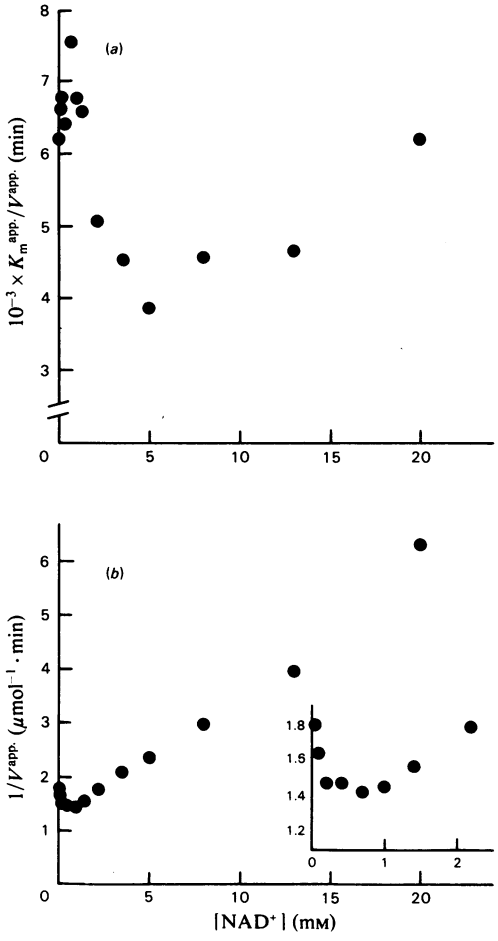


Fig. 3. *Variation of the Michaelis–Menten parameters for hydroxylamine with NAD$^+$ concentration*
The concentration of hydroxylamine was varied over the range 1–101mM. Identical quantities of the same enzyme preparation were used for each determination. The NADH concentration was 0.25mM.

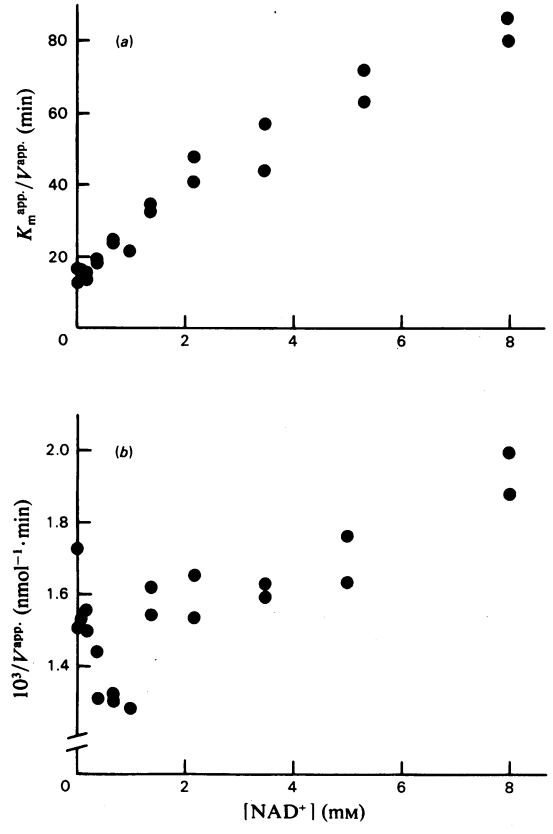


Fig. 4. *Variation of the Michaelis–Menten parameters for NADH with [NAD$^+$] with NO_2^- as oxidized substrate*
[NADH] was varied in the range 0.004–0.1mM for [NAD$^+$] up to 0.4mM and in the range 0.004–0.2mM for [NAD$^+$] 0.4mM and above. Identical quantities of the same preparation were used for each determination. The NO_2^- concentration was 1mM.

The reaction followed Michaelis–Menten kinetics for concentrations of hydroxylamine in the range 1–100mM, with slopes of Hill plots in the range 0.96–1.02. As [NAD^+] increased, the apparent value of K_m/V declined to a minimum at about 8mM-NAD^+ and then increased. There is also a suggestion of complex behaviour at low NAD^+ concentrations, but the accuracy of the data does not permit this to be characterized. The apparent value of $1/V$ also passed through a minimum, at the lower concentration of about 0.8mM-NAD^+, with very little suggestion of more complex behaviour.

Effect of NAD^+ on the reduction of nitrite

Coleman *et al.* (1978) reported that the apparent value of K_m for NADH measured in the presence of 1mM-NO_2^- showed a non-linear increase with the concentration of NAD^+, but were not able to detect corresponding variation in the apparent value of V. The more precise data obtained in the present study (Fig. 4) show that $1/V^{app.}$ increases with [NAD^+], though the exact nature of the dependence is not well defined. Within the limitations of the data it resembles the corresponding effect when hydroxylamine is substrate (Fig. 2*a*), and $K_m^{app.}/V^{app.}$ also increases with [NAD^+] in a similar way to that observed with hydroxylamine.

When NO_2^- was the varied substrate, at 0.25mM-NADH, $K_m^{app.}/V^{app.}$ decreased as [NAD^+] increased over the whole of the range considered (Fig. 5*a*), whereas $1/V^{app.}$ passed through a minimum at about 0.5mM-NAD^+ (Fig. 5*b*).

Although for both reactions the behaviour of NAD^+ as a product inhibitor is complicated by its effect as an activator at low concentrations, the inhibition characteristics are similar for both: NAD^+

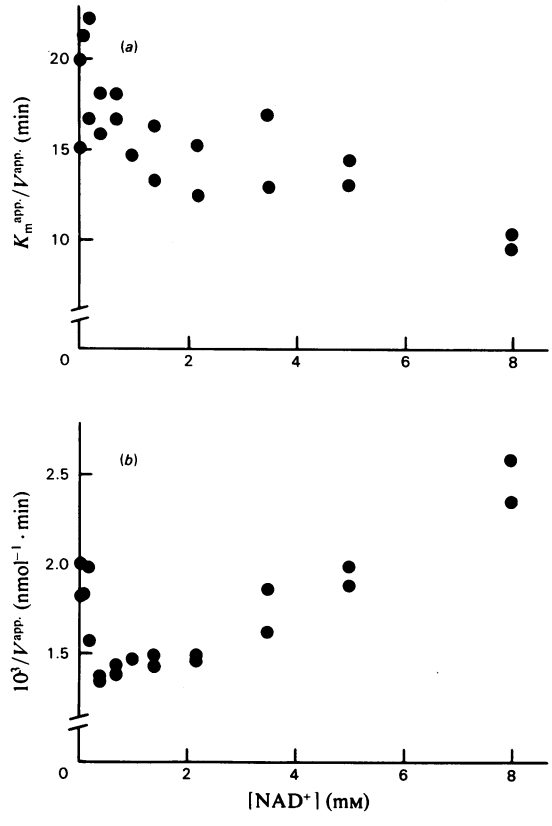


Fig. 5. *Variation of the Michaelis–Menten parameters for NO_2^- with [NAD^+]*

The concentration of NO_2^- was varied over the range 0.002–0.2mM. Identical quantities of the same preparation of enzyme were used for each determination. The NADH concentration was 0.25mM.

Table 2. *NAD^+ activates more than one step in the reduction of nitrite*

Apparent Michaelis–Menten parameters for nitrite and hydroxylamine were measured at three different concentrations of NAD^+ as specified, in the presence of 0.25mM-NADH and equal volumes of the same preparation of enzyme. Rates (of NADH oxidation) are given in nmol/min per ml of enzyme and V/K_m values are in min^{-1}. The calculation of the $k_{+2}e_0$ values shown in the right-hand column is based on Scheme 1 of Coleman *et al.* (1978), which supposes that reduction of both nitrite and hydroxylamine proceeds through the same enzyme–hydroxylamine complex, which breaks down to products with rate constant k_{+3} and is formed from a presumed enzyme–NO_2^- complex with net rate constant k_{+2}. This Scheme gives:

$$V^{app.}_{NH_2OH} = k_{+3}e_0 \quad \text{and} \quad V^{app.}_{NO_2^-} = 3k_{+2}k_{+3}e_0/(k_{+2}+k_{+3})$$

(Coleman *et al.*, 1978), which can be rearranged to give:

$$k_{+2}e_0 = 1/[(3/V^{app.}_{NO_2^-}) - (1/V^{app.}_{NH_2OH})].$$

	Hydroxylamine reduction		NO_2^- reduction		
[NAD^+] (mM)	$V^{app.} = k_{+3}e_0$	$V^{app.}/K_m^{app.}$	$V^{app.}$	$V^{app.}/K_m^{app.}$	$k_{+2}e_0$
0.05	883	0.264	392	39.0	153
0.7	1265	0.269	475	41.3	181
5.0	785	0.300	339	49.4	132

acts as a mixed inhibitor with respect to NADH in both reactions (Figs. 2 and 4), as a mixed inhibitor with respect to hydroxylamine (Fig. 3) and as an uncompetitive inhibitor with respect to NO_2^- (Fig. 5), though in this last case it is possible that a competitive component in the inhibition would have been observed at higher NAD^+ concentrations.

Activation of NO_2^- reduction by NAD^+ also resembled the activation of hydroxylamine reduction, with the highest values of $V^{app.}$ observed over the same range of NAD^+ concentrations (Table 2). It is plausible that NAD^+ both activates and inhibits the enzyme at the same site at which NADH binds to reduce it.

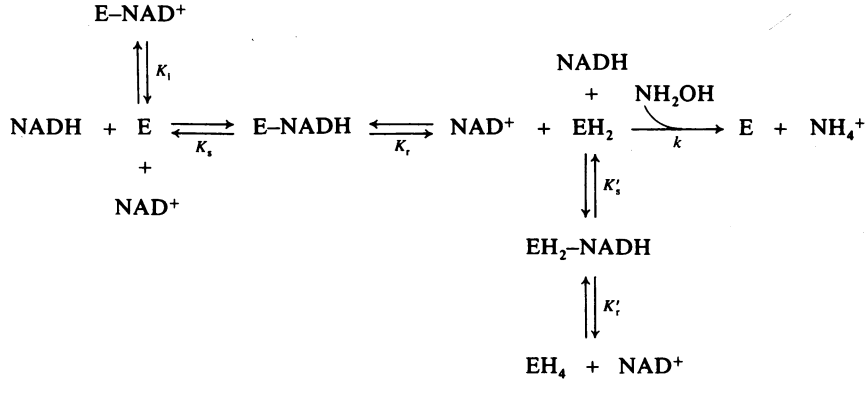
Discussion

Nitrite reductase contains three types of prosthetic group: FAD, iron–sulphur centres and sirohaem (Jackson *et al.*, 1981). It is likely that NADH reduces the enzyme at the flavin and that hydroxylamine and NO_2^- bind to sirohaem and therefore that the binding site for NADH is spacially separated from that for hydroxylamine and NO_2^-. If this is so, reduced substituted-enzyme species must be formed during catalysis. The inhibition characteristics are consistent with this type of mechanism if a transition between enzyme forms (perhaps involving electron transfer between the prosthetic groups of the enzyme) occurs after NAD^+ release but before binding of hydroxylamine. If NAD^+ also inhibits by competing with NADH for binding to oxidized enzyme, the observed inhibition can be explained without the need for such a transition. Indeed, the results are consistent with the binding of oxidized substrate being independent of reduction of the enzyme, as has been suggested for several multicentre redox enzymes (e.g. xanthine dehydrogenase, Coughlan & Rajagopalan, 1980). However, for the sirohaem-containing enzymes *E. coli* sulphite reductase and *Cucurbita pepo* nitrite reductase, reduction of the enzyme is essential for the rapid binding of oxidized substrate (Rueger & Siegel, 1976; Cammack *et al.*, 1978).

Models for the mechanism of the NADH-dependent reduction of hydroxylamine

The simplest type of model to explain NAD^+ activation is one in which excess NADH inhibits the enzyme by reducing it to an inactive form and NAD^+ activates by reversing this over-reduction. Scheme 1 shows a model of this type for NADH oxidation in the presence of saturating hydroxylamine. This scheme is analogous to that proposed by Matthews *et al.* (1976) for lipoamide dehydrogenase. The first molecule of NADH to bind reduces the oxidized enzyme E to a two-electron reduced form EH_2 and NAD^+ is released. EH_2 donates

Scheme 1. *Model for the oxidation of NADH by hydroxylamine*



This model represents an attempt to explain activation and inhibition of nitrite reductase by NAD^+ in terms of a single NAD-binding site, and supposes that NAD^+ activates by antagonizing over-reduction of the enzyme by NADH. If all steps except the oxidation of EH_2 by NH_2OH were equilibria with the equilibrium constants shown, this model would give a rate equation of the form:

$$v = \frac{ke_o}{1 + \frac{[NAD^+]}{K_r} + \frac{[NADH]}{K'_s} + \frac{K_s[NAD^+]}{K_r[NADH]} + \frac{K'_r[NADH]}{K'_s[NAD^+]} + \frac{K_s[NAD^+]^2}{K_rK_i[NADH]}}$$

where e_o is the total enzyme concentration and k is the rate constant for the reaction of EH_2 with hydroxylamine at saturating [hydroxylamine].

electrons to hydroxylamine to form NH_4^+. Alternatively, a second molecule of NADH can reduce EH_2 to a four-electron reduced form that has negligible catalytic activity. NAD^+ inhibits both as a product inhibitor by reoxidizing EH_2 to E and as a dead-end inhibitor by competing with NADH for the free enzyme E. Substrate inhibition results from over-reduction of the active enzyme EH_2 by NADH to give an inactive form EH_4, and activation by NAD^+ arises because NAD^+ antagonizes this over-reduction. The rate equation predicted by this model under simplifying assumptions is shown in the legend to the Scheme and is consistent with the data shown in Figs. 2 and 3. Approximate adherence of the data to this equation does not of course exclude a more complex interpretation.

Scheme 1 requires only a single site that binds both NADH and NAD^+. Other models can, however, account for the pattern of the variation of the Michaelis–Menten parameters for hydroxylamine and NADH with $[NAD^+]$, including models in which some or all of the equilibrium assumptions are not made. It is possible that there are two NAD binding sites and that NADH inhibits by saturating an inhibitory site distinct from the active site, possibly but not necessarily by over-reducing the enzyme. NAD^+ would activate by competing with NADH for this inhibitory site. There are, however, a number of advantages in adopting a model with only one NAD binding site, such as that in Scheme 1. First, the nitrite reductase subunit (M_r 88000) may be too small to contain two NAD binding sites as well as FAD, sirohaem and iron–sulphur centres. Second, there is strong evidence for NAD^+ activation of lipoamide dehydrogenase by reversal of an over-reduction at the active site (Matthews *et al.*, 1976). In particular, NAD^+ activation can be understood simply in terms of a single site that binds both NAD^+ and NADH by comparing the redox potentials of the couples E/EH_2 and EH_2/EH_4 with that of the NAD^+/NADH couple (Matthews & Williams, 1976). Third, to produce models with two NAD^+ binding sites that can account for all the data it is necessary to rule out the formation of chemically reasonable species such as $E(NAD^+)_2$.

The behaviour of hydroxylamine as a substrate for nitrite reductase and its stability are consistent with its occurrence as an enzyme-bound intermediate in the reduction of NO_2^-. Coleman *et al.* (1978) accordingly suggested that an enzyme–hydroxylamine complex was produced by four-electron reduction of NO_2^-. This would require the binding of hydroxylamine to the free enzyme to proceed with an on rate constant no more than 1.6% of the corresponding rate constant for binding NO_2^-, but this is consistent with the fact that hydroxylamine is not a natural substrate for the enzyme and cannot be detected as a product during NO_2^- reduction. Any energy barrier to prevent premature release of hydroxylamine would also oppose its binding.

The dependence of the apparent maximum velocity on $[NAD^+]$ is less marked for NO_2^- as substrate than for hydroxylamine. Coleman *et al.* (1978) suggested that this might be because NAD^+ activation of the enzyme derived solely from activation of the breakdown of the enzyme–hydroxylamine complex. To test this hypothesis we measured the kinetic parameters for both hydroxylamine and NO_2^- with 0.25mM-NADH using the same preparation of enzyme at three concentrations of NAD^+ (Table 2).

By means of the relationships given by Coleman *et al.* (1978) (and quoted in the legend to Table 2) one can calculate the maximum rates of conversion of enzyme–NO_2^- complex into enzyme–hydroxylamine complex from the observed apparent maximum velocities for the two substrates, with the results shown in the right-hand column of Table 2. The observed variation with $[NAD^+]$ shows that the activation by NAD^+ is more complex than postulated previously and that NAD^+ does activate at least one step before formation of the enzyme–hydroxylamine complex. Conceptually the simplest interpretation of this is that NAD^+ activates each of the three NADH-dependent reductions in the same way, by preventing over-reduction of the enzyme, and to the same degree. The observation that activation of NO_2^- reduction is less pronounced than activation of hydroxylamine reduction would be consistent with this interpretation, provided that there is at least one step between NO_2^- and hydroxylamine that is not affected by NAD^+ and is slow enough to affect the net rate.

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