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

The reduction of single-electron acceptors

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The kinetic characteristics of the diaphorase activities associated with the NADH-dependent nitrite reductase (EC 1.6.6.4) from *Escherichia coli* have been determined. The values of the apparent maximum velocity are similar for the reduction of Fe(CN)$_6^{3-}$ and mammalian cytochrome c by NADH. These reactions may therefore have the same rate-limiting step. NAD$^+$ activates NADH-dependent reduction of cytochrome c, and the apparent maximum velocity for this substrate increases more sharply with the concentration of NAD$^+$ than for hydroxylamine. The simplest explanation is that NAD$^+$ activation of hydroxylamine reduction derives solely from activation of steps involved in the reduction of cytochrome c, a flavin-mediated reaction, but these steps are only partly rate-limiting for the reduction of hydroxylamine. At 0.5 mM-NAD$^+$, the apparent maximum velocity was 2.3 times higher for 0.1 mM-cytochrome c as substrate than for 100 mM-hydroxylamine, suggesting that the rate-limiting step during hydroxylamine reduction is a step that is not involved in cytochrome c reduction. A scheme is proposed that can account for the pattern of variation with [NAD$^+$] of the Michaelis–Menten parameters for hydroxylamine and for NADH with hydroxylamine or cytochrome c as oxidized substrate.

The NADH-dependent nitrite reductase from *Escherichia coli* K12 (NADH: nitrite oxidoreductase, EC 1.6.6.4) catalyses both the six-electron reduction of nitrite and the two-electron reduction of hydroxylamine to ammonia (Coleman et al., 1978). Prosthetic groups include sirohaem, FAD and one or more iron–sulphur centres (Jackson et al., 1981a). The enzyme is activated by the oxidized product NAD$^+$, but inhibited by high concentrations of the reduced substrate, NADH. These kinetic characteristics are consistent with a model that postulates that there is a single pyridine nucleotide binding site: according to this model, NAD$^+$ activates by antagonizing over-reduction of the enzyme by NADH (Scheme 1 in Jackson et al., 1981b).

Nitrite reductase also catalyses the reduction of the single-electron acceptors horse-heart cytochrome c, dichlorophenolindophenol and K$_3$Fe(CN)$_6$ by NADH. These activities are independent of the haem group (Jackson et al., 1981a). Similar 'diaphorase' activities are detected with other flavoproteins that are specific for reduced pyridine nucleotides, and these activities are also mediated by the flavin prosthetic group (Massey et al., 1969).

According to the scheme postulated by Jackson et al. (1981b), the rate-limiting step for nitrite reduction occurs after the formation of the two-electron-reduced enzyme species, EH$_2$: if this is correct, the diaphorase activity of nitrite reductase should be more sensitive than the rates of reduction of nitrite or hydroxylamine to changes in the concentrations of NAD$^+$ and NADH. A kinetic analysis of the diaphorase activity has therefore been undertaken to test this prediction and to provide further insight into the sequence of electron transfer reactions during the reduction of nitrite or hydroxylamine.

Materials and methods

Enzyme and substrate

Nitrite reductase was purified from *E. coli* strain OR75Ch15 as described by Jackson et al. (1981a),

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and appeared to be more than 90% pure as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Horse-heart cytochrome c (grade 3) was obtained from Sigma.

**Measurement of NADH-dependent 'diaphorase' activities**

Open sample and reference cuvettes (1.5 ml) contained various concentrations of NADH, NADH and cytochrome c or K$_2$Fe(CN)$_6$ in 1 ml of buffer [50 mM-Tris/HCl (pH 8.0)/5 mM-EDTA] at 30°C. The reaction was initiated by adding the enzyme to the sample cuvette. 'Diaphorase' activities were calculated from the rate of change of absorbance at 340 nm for reduction of K$_2$Fe(CN)$_6$ or at 550 nm for reduction of cytochrome c. Rates were corrected for the non-enzymic reduction of Fe(CN)$_6^{3-}$ by NADH. Initial rates were calculated from orthogonal polynomials fitted to the progress curves (Booman & Niemann, 1956). The molar absorbance coefficient for reduced minus oxidized cytochrome c was taken to be $e_{340} = 2.1 \times 10^3$ M$^{-1}$cm$^{-1}$ (Masters et al., 1965).

For each determination of Michaelis–Menten parameters, eight concentrations of the varied substrate were used, three of the determinations being in duplicate. Values of the parameters were calculated as in Jackson et al. (1981b). The ranges of substrate concentrations used were 3–100 $\mu$M for cytochrome c, 0.1–2.0 mM for Fe(CN)$_6^{3-}$, 5–200 $\mu$M for NADH when NH$_2$OH was the oxidant and from 5 $\mu$M to a concentration above which deviation from Michaelis–Menten kinetics was readily apparent with cytochrome c as oxidant.

Each point in the Figures represents a single determination, the same volume of the same preparation being used for all determinations.

**Results and discussion**

The peak of NADH-dependent cytochrome c reductase eluted from the DEAE-Sephadex column in the last step of the purification procedure was completely coincident with the peak of nitrite reductase activity (Fig. 1); the ratio of the two activities across the peak was 5.56 $\pm$ 0.16. By the criterion of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the enzyme eluted from this column was 95% pure and no single contaminant accounted for more than 1% of the total protein. It follows that the cytochrome c reductase activity is almost certainly due to nitrite reductase rather than to a contaminant, because such a contaminant, if it existed, would need to have a catalytic constant of at least $1.4 \times 10^{4}$ s$^{-1}$ to account for the observed behaviour.

To establish the stoichiometry of the reduction of cytochrome c by NADH, the amount of NADH oxidized during the reduction of a fixed amount of cytochrome c was measured by the change in absorbance at 340 nm and compared with the change in absorbance at 550 nm. Results were corrected for the amount of NADH oxidized in the absence of cytochrome c. For every NADH molecule oxidized, 1.96 $\pm$ 0.08 (five determinations, s.e.m.) molecules of cytochrome c were reduced.

The values of $K_m^{app}$ and $V^{app}$ for reduction of cytochrome c and Fe(CN)$_6^{3-}$ were determined with 0.2 mM-NADH and 0.7 mM-NAD$^+$ (Table 1). The similar values of $V^{app}$ for the reduction of cytochrome c and Fe(CN)$_6^{3-}$ suggest that the rate-limiting step is the same for the two reactions. Furthermore, similar rates of oxidation of 0.2 mM-NADH were obtained with either 1 mM-Fe(CN)$_6^{3-}$ or 0.1 mM-cytochrome c, when the NAD$^+$ concentration was 0.7, 1.0 or 5.0 mM.

The addition of 1 mM-N0$_2^-$ to assay mixtures did not significantly lower the rate of NADH-dependent reduction of cytochrome c, as measured by the change in absorbance at 550 nm (Table 2). Further, the rate of NADH oxidation with 1 mM-N0$_2^-$ and 1 mM-Fe(CN)$_6^{3-}$ is the same as, or a little more than, the rate with 1 mM-Fe(CN)$_6^{3-}$ alone. If N0$_2^-$ competed for the same site as cytochrome c and Fe(CN)$_6^{3-}$, it would be expected to have an apparent inhibition constant equal to its $K_m$ (Cornish-Bowden, 1979), and hence to decrease the rate of NADH-dependent reduction of cytochrome c by 77% and that of Fe(CN)$_6^{3-}$ by 90%. That this was not observed confirms the conclusion from the effects of
Kinetics of nitrite reductase

Table 1. Kinetic constants for diaphorase reactions
All kinetic constants were determined in the presence of 0.7 mM-NAD+. Reactions were initiated with 20 μl samples of enzyme (5 nkat of nitrite reductase/ml when hydroxylamine was the oxidized substrate; 1 nkat/ml for cytochrome c reduction). Values of \( V^{\text{app}} \) have been corrected for this difference in enzyme concentration. Abbreviation: n.d., not determined with this enzyme preparation.

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>Constant substrate</th>
<th>( K_m^{\text{app}} (\mu M) )</th>
<th>( V^{\text{app}} ) [μmol of NADH oxidized·min^{-1}·(ml enzyme)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>0.2 mM-NADH</td>
<td>3.9</td>
<td>27.9</td>
</tr>
<tr>
<td>( K_{i, \text{Fe(CN)}_6} )</td>
<td>0.2 mM-NADH</td>
<td>100</td>
<td>29.8</td>
</tr>
<tr>
<td>NADH</td>
<td>0.1 mM-cytochrome c</td>
<td>48.8</td>
<td>34.4</td>
</tr>
<tr>
<td>NO(_2^)</td>
<td>0.2 mM-NADH</td>
<td>11.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>NH(_2)OH</td>
<td>0.2 mM-NADH</td>
<td>2910</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Table 2. Competition between diaphorase acceptors and nitrite during NADH-dependent reduction
All reaction mixtures contained 0.7 mM-NAD+.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrates added</th>
<th>Rate ± standard deviation [μmol of NADH oxidized·min^{-1}, (ml of enzyme)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of NO(_2^) on cytochrome c reduction measured at 550 nm</td>
<td>200 μM-NADH, 0.1 mM-cytochrome c</td>
<td>36.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>200 μM-NADH, 0.1 mM-cytochrome c, 1 mM NO(_2^)</td>
<td>35.9 ± 0.7</td>
</tr>
<tr>
<td>Competition between Fe(CN)(_6)(^{3-}) and NO(_2^) for NADH oxidation, measured at 340 nm</td>
<td>200 μM-NADH, 1 mM NO(_2^)</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>200 μM-NADH, 1 mM Fe(CN)(_6)(^{3-}), 1 mM NO(_2^)</td>
<td>42.1 ± 1.5</td>
</tr>
<tr>
<td>Competition between cytochrome c and NO(_2^) for NADH oxidation, measured at 340 nm</td>
<td>100 μM-NADH, 1 mM NO(_2^)</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>100 μM-NADH, 0.1 mM-cytochrome c</td>
<td>33.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>100 μM-NADH, 0.1 mM-cytochrome c, 1 mM NO(_2^)</td>
<td>33.7 ± 2.1</td>
</tr>
</tbody>
</table>

CN\(^-\) (Jackson et al., 1981a) that NO\(_2^\) and cytochrome c or Fe(CN)\(_6\)\(^{3-}\) accept electrons at separate sites. It also suggests that the inhibition constant for NO\(_2^\) is very high and that a slow step occurs during nitrite reduction after the formation of the reduced enzyme species that donates electrons to cytochrome c or Fe(CN)\(_6\)\(^{3-}\).

Effect of NAD\(^+\) on the rate of cytochrome c reduction by NADH

The rate of cytochrome c reduction was determined by measuring the rate of increase in absorbance at 550 nm with 200 μM-NADH and 0.1 mM-cytochrome c but no added NAD\(^+\). The rate was not constant but increased with time, increasing by 40% in the first 30 s after mixing. A similar acceleration was observed when the rate of oxidation of NADH by NO\(_2^\) or NH\(_2\)OH was monitored (Coleman et al., 1978). This activation of cytochrome c reduction by NAD\(^+\) is, however, the first direct demonstration that the formation of reduced product accelerates concomitantly with oxidation of NADH. As was observed for the reduction of NO\(_2^\) and hydroxylamine (Jackson et al., 1981b), NADH is a substrate inhibitor of cytochrome c reduction. A plot of the rate of reaction against [NADH] at 0.05 mM-NAD\(^+\) and 0.1 mM-cytochrome c deviated from the pattern predicted by the Michaelis–Menten equation, the rate decreasing as [NADH] increased from 200 μM to 800 μM.

The effect of NAD\(^+\) on the Michaelis–Menten parameters for NADH was investigated with 0.1 mM-cytochrome c as constant substrate. Over the range 0.02–0.5 mM, \( V^{\text{app}} \) increased sharply before reaching a plateau or slowly declining at higher [NAD\(^+\)] (Fig. 2a). \( K_m^{\text{app}} \) for NADH increased non-linearly with [NAD\(^+\)] (Fig. 2b), as did \( K_m^{\text{app}} / V^{\text{app}} \). Thus, the variation of \( K_m^{\text{app}} \) and \( K_m^{\text{app}} / V^{\text{app}} \) for NADH with [NAD\(^+\)] was qualitatively similar to that when 100 mM-hydroxylamine was the oxidized substrate (Jackson et al., 1981b).

There were three striking differences in the behaviour of the Michaelis–Menten parameters for NADH when 0.1 mM-cytochrome c rather than 100 mM-hydroxylamine was the oxidized substrate. First, the values of \( V^{\text{app}} \) for oxidation of NADH were much higher for oxidation of cytochrome c than for oxidation of hydroxylamine (Fig. 2a). This may be due to an extra rate-limiting step during hydroxylamine reduction that is not involved in cytochrome c reduction. Alternatively, there may be nitrite reductase molecules that lack sirohaem in
haem content of nitrite reductase was not determined by a rigorous method, however, independent confirmation of the presence of two slow steps during hydroxylamine reduction is required. The molar catalytic activity of the NADPH-dependent sulphite reductase of *E. coli* (which also contains sirohaem) for cytochrome *c* reduction is also substantially higher than for reduction of hydroxylamine, NO$_2^-$ or SO$_3^{2-}$ (Siegel et al., 1974). For this enzyme, the reduction of FAD by NADPH appears to be rate-limiting for the NADPH-dependent reduction of cytochrome *c* (Siegel et al., 1971). Two further steps, electron transfer between flavin and haem, and release of S$^2$ from sirohaem appear sufficiently slow to be rate-limiting for SO$_3^{2-}$ reduction (Siegel & Kamin, 1968; Rueger & Siegel, 1976).

The second difference from the behaviour with hydroxylamine was that activation by NAD$^+$ is more pronounced for the reduction of cytochrome *c*. Thus, $V^{\text{app}}$ for cytochrome *c* reduction increases by 80% on increasing the NAD$^+$ concentration from 0.025 mM to 0.5 mM, whereas for hydroxylamine reduction it increases by only 41% over the same range. The simplest explanation is that NAD$^+$ activation of hydroxylamine reduction derives solely from activation of steps involved in the reduction of cytochrome *c*, a flavin-mediated reaction that is not dependent on the haem and is only partially rate-limiting in the reduction of hydroxylamine.

The third difference in the behaviour with cytochrome *c* is that the sharp decline in $V^{\text{app}}$ for NADH between 0.5 mM-NAD$^+$ and 5 mM-NAD$^+$ seen with hydroxylamine as oxidized substrate does not occur with 0.1 mM-cytochrome *c*. Although this may be because cytochrome *c* reduction follows a different type of mechanism from hydroxylamine reduction, the common features of the two activities suggest that the mechanisms are similar: both activities are activated by NAD$^+$, inhibited by the substrate NADH and show qualitatively similar variation of $K_m^{\text{app}}$ and $K_m^{\text{app}}/V^{\text{app}}$ for NADH with NAD$^+$.

**Model for the mechanism of the NADH-dependent reduction of cytochrome *c* and hydroxylamine**

A model similar to Scheme 1 of Jackson et al. (1981b) can be applied to the NADH-dependent reduction of cytochrome *c* (Scheme A). Again, NADH can ‘over-reduce’ the enzyme to an inactive form EH$_4$. NAD$^+$ inhibits as a product inhibitor by reoxidizing EH$_4$ to E and by competing with NADH for binding to E. NAD$^+$ activates by reoxidizing EH$_4$ to EH$_2$. Scheme A differs from Scheme 1, however, in not assuming that the steps forming EH$_2$ are at equilibrium. Thus it does not assume that the formation of EH$_2$ is much faster than the rate of
reoxidation of EH₂ by cytochrome c, rate constant k⁺₁. Indeed, it is rare for electron transfer to the oxidant to be rate-limiting in multi-centre redox enzymes (Palmer & Olson, 1980), and so it is more likely that k⁺₁ is much greater than k₋ᵣ, the rate constant for the reduction of nitrite reductase by NAD⁺ to give EH₂ with release of the oxidized product NAD⁺.

The equation derived from this model for the reduction of cytochrome c by NAD⁺ is as follows:

\[
v = \frac{k₊ₑₒ}{1 + \frac{Kₛ \cdot \left[\text{NADH}\right]}{\left[\text{NADH}\right] \cdot k₊₁ + \frac{\left[\text{NAD⁺}\right] \cdot k₋ᵣ}{k₋ᵣ}}}
\]

It is consistent with the observed non-linear increase in \(Kₘ^{app}\) for NADH with increasing [NAD⁺] and with the apparent deviation from linearity in the increase with [NAD⁺] of \(Kₘ^{app}/V^{app}\) for NADH. The plateau or only marginal decline in \(V^{app}\) for NADH at high [NAD⁺] is consistent with the model if \(k₊₁ ≫ k₋ᵣ\) for [NAD⁺] up to 10 mM.

The equation derived from Scheme A for the dependence of the rate of NADH oxidation on [NAD⁺] and [NAD⁺] at 100 mM-hydroxylamine is the same as the above equation with \(k₊₁\) replaced by \(k₊ₜₜ\) throughout. It is of the same form as the equation derived from Scheme 1 of Jackson et al. (1981b); it therefore predicts the same pattern of variation in the apparent Michaelis–Menten parameters for NADH with [NAD⁺] and is compatible with the observed inhibition characteristics of NAD⁺ with respect to NADH (Jackson et al., 1981b). The rate constant \(k₊ₜₜ\) is the rate constant for the slowest step of the following four: (i) the intra-molecular transfer of electrons to sirohaem in the two-electron reduced form EH₂; (ii) the binding of hydroxylamine to sirohaem; (iii) the reduction of hydroxylamine; (iv) release of NH₄⁺. Scheme A can explain the sharp decline in \(V^{app}\) for NADH when hydroxylamine is the oxidized substrate (Jackson et al., 1981b; Fig. 2a) if the rate constant \(k₊ₜₜ\) is not much greater than \(k₋ᵣ\) [NAD⁺]. An increase in [NAD⁺] will lower \(V^{app}\) for NADH

by driving the reaction E-NADH = EH₂ + NAD⁺ to the left and therefore lowering the concentration of EH₂ in the steady state. As noted above, if Scheme A applies, \(k₊₁ ≫ k₋ᵣ\) [NAD⁺] for [NAD⁺] up to 10 mM. This model therefore suggests that \(k₊ₜₜ\) is substantially lower than \(k₊₁\). The data are compatible with E-NADH = EH₂ + NAD⁺ being rate-limiting for cytochrome c and hydroxylamine reduction. If, additionally, the rate of re-oxidation of EH₂ by hydroxylamine was slow enough to limit the overall rate of hydroxylamine reduction, then the model would still be consistent with the data.

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References