Suicide inactivation of xanthine oxidoreductase during reduction of inorganic nitrite to nitric oxide

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INTRODUCTION

The molybdoflavoenzyme, xanthine oxidoreductase (XOR), occurs widely in Nature, and has been studied intensively [1], being readily available in cows’ milk, where it is a major protein component of the milk fat globule membrane [2,3]. XOR occurs as a homodimer; each 147 kDa subunit contains one mol-
XDH was prepared by incubating XO in the presence of 10 mM dithiothreitol at 37.0 ± 0.2 °C for 1 h, followed by gel filtration on a PD-10 desalting column (Pharmacia). XDH prepared in this manner contained 10–15% oxidase activity, as determined by the method described below.

Concentrations of enzyme were determined from the UV-visible spectrum, by using an absorption coefficient of 36 M^−1 cm^−1 (of subunit) cm^−1 at 280 nm [2]. The oxidase contents of XO or XDH were determined by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically at 295 nm in a Cary 100 spectrophotometer, using an absorption coefficient of 9.6 M^−1 cm^−1 [38]. Assays were performed at 25.0 ± 0.2 °C in air-saturated Na-Bicine buffer, pH 8.3, containing 100 μM xanthine. The sum of oxidase and dehydrogenase contents was determined as above but in the presence of 0.5 mM NAD^+.

Iodonium diphenyl (IoDP)-inhibited enzyme

IoDP-inhibited XO was prepared essentially as described by O’Donnell et al. [39] and outlined by Godber et al. [18].

Reagents

Dithiothreitol was purchased from Alexis Corp. Oxygen-free nitrogen and compressed air were from British Oxygen Corp. All other reagents, unless otherwise stated, were purchased from Sigma.

Kinetics of XOR-catalysed oxidation of xanthine or NADH in the presence of inorganic nitrite or nitrate

All kinetic studies were carried out under anaerobic conditions in nitrogen-sparged 50 mM potassium phosphate buffer, pH 7.2, at 25.0 ± 0.2 °C using an anaerobic cabinet (Belle Technologies) containing < 3 p.p.m. O2. XOR was mixed with 100 μM xanthine or 100 μM NADH and various concentrations of sodium nitrite or nitrate, using a Hi-Tech SF61 rapid-mixing device. With xanthine as reducing substrate, urate was determined as described above for determination of oxidase content of XOR. With NADH as reducing substrate, NADH utilization was followed at 340 nm using an absorption coefficient of 6.22 M^−1 cm^−1 [40].

Fluorescence assay for XOR activity

The sensitive assay of Beckman et al. [41] was used for samples with low XOR activity. The assay utilizes the enzyme-catalysed oxidation of pterin to isoxanthopterin, an oxidation totally analogous to that of xanthine to urate. Assays were carried out in a Perkin-Elmer LS-50B luminescence spectrometer at 25.0 ± 0.2 °C, and isoxanthopterin was detected fluorimetrically with an excitation wavelength of 345 nm and emission wavelength of 390 nm. Sample was added to 50 mM potassium phosphate, pH 7.2, in 1 ml quartz fluorimeter cells and fluorescence was monitored to ensure a stable baseline. Pterin was added to a final concentration of 10 μM, mixed by inversion and the rate of fluorescence change was determined. Confirmation that the generation of product was allopurinol-inhibitable was obtained by addition of allopurinol to a final concentration of 10 μM. The system was calibrated by adding known amounts of isoxanthopterin, which acts as an internal standard and takes into account the differences observed in fluorescence quenching and light scatter.

Inactivation of XOR in the presence of inorganic nitrite or nitrate

XOR was incubated in an anaerobic cabinet, as described above, with various concentrations of sodium nitrite or nitrate and xanthine. Aliquots (400 μl) of the reaction mixture were taken at intervals, gel filtered on a PD-10 desalting column (Pharmacia) and assayed fluorimetrically for enzyme activity, as described above. In larger-scale experiments, where spectral changes of the enzyme were also monitored, the spectrophotometric urate assay was used (see above). These latter experiments were carried out at 20 °C to allow sufficient time to record enzyme activities and spectra. As the enzyme concentrations in the PD-10 eluates were not identical, the spectra were normalized using the ratio of the absorbances at 450 and 650 nm.

UV-visible spectra of XO before and after inactivation

XO (2.1 μM) was incubated under anaerobic conditions (see above) for 1 h, in the absence and presence of 1 mM xanthine and 80 mM sodium nitrite. Samples of enzyme were gel filtered to remove substrates and product and the UV-visible spectra were recorded using a Cary 100 spectrometer. XOR incubated in the absence and presence of xanthine and sodium nitrite showed specific activities of 1620 and 48 nmol of urate·min^−1·mg^−1 respectively, the latter representing 3% of the former.

Use of NO electrode for inhibition kinetics

NO was measured by using a specific electrode (ISO-NO World Precision Instruments) in a water-jacketed, stirred chamber. The tip of the electrode was submerged 5 mm into the reaction buffer. Reactions (4 ml) were carried out in nitrogen-sparged 50 mM potassium phosphate, pH 7.2, at 25.0 ± 0.2 °C in an anaerobic cabinet and started by addition of enzyme. Readings of current were taken at 5 s intervals and plotted against time to obtain initial rates. The electrode was calibrated under the conditions of the assay by using a standard NO-generating system (ISO-NO Technical Manual), whereby known quantities of sodium nitrite were added to 0.1 M sulphuric acid, containing 0.1 M potassium iodide, to give stoichiometric amounts of NO. Recorded currents were plotted against concentrations of NO to give a linear standard curve.

Determination of nitrite concentrations

Nitrite concentrations were determined by using the Griess reagent system (Promega, Madison, WI, U.S.A.), which relies on a diazotization reaction between sulphanilamide and N-1-naphthylene diamine dihydrochloride under acid conditions [42]. In the presence of nitrite, a magenta colour develops which is monitored at 535 nm. Nitrite concentration was determined from a standard reference curve in the range 0–200 μM.

Analysis of kinetic data

The fitting of the kinetic model of Scheme 1 (see below) to time courses of inactivation and to progress curves was carried out using Scientist software (MicroMath), employing non-linear least-squares minimization of the numerically integrated rate equations utilizing Powell’s algorithm. Characterization of steady-state inhibition was carried out using the complementary methods of Dixon [43] and Cornish-Bowden [44].
RESULTS
In the presence of nitrite and xanthine, XO is progressively inactivated by conversion into its desulpho-form

We have previously reported that the incubation of XO with inorganic nitrate and xanthine under anaerobic conditions generates NO and that the enzyme is progressively inactivated [18]. After prolonged incubation, XO was shown to have been converted into its desulpho-form. This was demonstrated by its reactivation under standard resulphuration conditions, involving anaerobic incubation of dithionite-reduced enzyme with sulphide [18]. That the inactivated enzyme, so obtained, was indeed desulpho-XO was confirmed by determination of its UV-visible spectrum (Figure 1), which is characteristic of this form [45]. As noted previously [45], the difference spectrum between desulpho- and sulpho-XO shows two minima, at 320 and 437 nm. Monitoring of absorbance changes at 320 nm allowed the sulpho-desulpho conversion to be followed during the course of incubation of XO with xanthine and nitrite. By these means, the conversion was shown to be concurrent with inactivation of the enzyme (Figure 2).

Dependence of XO inactivation rate on nitrite concentration

In order to clarify the mechanisms underlying the above inactivation, the loss of enzyme activity was followed over time at various initial nitrite concentrations. The results are shown in Figure 3, in which it is seen that the rate of activity loss increases with increasing nitrite concentration.

Two kinetic mechanisms can be proposed that might account for the above inactivation. The first is based on the findings of Ichimori et al. [36], who reported that XO activity of XOR is abolished during incubation of the enzyme with NO. These workers found that the kinetics of inactivation were pseudo first order in enzyme, with a second-order rate constant of 14.8 M$^{-1}$·s$^{-1}$. They also proposed that inactivation only occurs in the presence of reducing substrate and in the absence of oxygen, implying that the inactivation process involves a reduced species of XOR. In the current study, NO generated during the incubation [18] could then inactivate reduced enzyme. This mechanism can be formulated as a two-stage process where E + S $\rightarrow$ ES $\rightarrow$ E + P occurs concurrently with E + P $\rightarrow$ E$_{ox}$ where E$_{ox}$ is inactivated enzyme. Such a mechanism would be expected to show a lag in the inactivation process, as the product concentration increases with time. Attempts to fit this mechanism to the activity decay data (Figure 3, dashed lines) do indeed display a sigmoid shape. As can be seen, the fit is poor.

A possible alternative is based on a mechanism-induced inactivation process (sometimes termed ‘suicide’ inactivation). This would occur in the enzyme–substrate (or enzyme–product) complex during turnover of substrate to product. At any fixed substrate concentration (i.e. assuming no substrate depletion), such a mechanism would be expected to show a simple monotonic exponential decay of activity with time.

The good fits of the experimental data to monotonic decay curves (Figure 3) suggest that a suicide mechanism of inactivation is likely. However, it is possible that there is a contribution from the product-based inactivation process, giving rise to a small initial lag in the inactivation curve. Such a small lag might be missed because of potential inaccuracies at early time points, resulting from the need to gel filter samples prior to assay of remaining enzyme activity. Gel filtering is necessary to avoid reaction of residual NO with superoxide, generated in the aerobic assay, to give peroxynitrite and consequent further inactivation [34,35].

Progress curves of XOR-catalysed oxidation of xanthine in the presence of nitrite

Confirmation of the existence of the suicide mechanism was sought from kinetic analysis of the progress curves for the enzyme-catalysed anaerobic reduction of nitrite to NO by xanthine. Such analysis should provide independent evidence for the suicide mechanism, and also allow quantitative assessment of the contribution of each of the two mechanisms to the inactivation process, as well as supplying accurate estimates of the kinetic parameters involved.

A minimal kinetic model for the XOR-catalysed reduction of inorganic nitrite, which incorporates both inactivation by product and suicide inactivation, is shown in Scheme 1. Here, E$_{ox}$...
Figure 3 Dependence of decay of enzymic activity on time and nitrite concentration

XO (10 nM active subunits) was incubated under anaerobic conditions with 100 μM xanthine and 15 mM (●), 30 mM (○) or 60 mM (■) sodium nitrite. The percentage of initial enzymic activity is plotted against time. Values are means ± S.D. (n = 3) of samples taken from individual experiments. The solid lines represent the fits to a single exponential decay curve. Dashed lines are fits to a mechanism involving inactivation by accumulated product (see the Results section).

Scheme 1 A minimal kinetic model for the XOR-catalysed reduction of inorganic nitrite, incorporating both inactivation by product and suicide inactivation

\[ \text{E}_\text{OX} + \text{xanthine} \xrightarrow{k} \text{E}_\text{R} + \text{urate} \]

\[ \begin{align*}
\text{E}_\text{X} &\xrightarrow{k_i} + \text{NO} \\
\text{E}_\text{OX} \cdot \text{NO}_2^- &\xrightarrow{k_i} \text{E}_\text{X} \cdot \text{NO}_2^- \\
\text{E}_\text{R} \cdot \text{NO}_2^- &\xrightarrow{k_i} \text{E}_\text{X} \cdot \text{NO}_2^- \\
\text{E}_\text{R} \cdot \text{NO}_2^- &\xrightarrow{k_{\text{cat}}} \text{E}_\text{OX} \\
\end{align*} \]

represents oxidized enzyme, \( \text{E}_\text{R} \) is reduced enzyme and \( \text{E}_\text{X} \) is inactivated enzyme. \( \text{E}_\text{R} \cdot \text{NO}_2^- \) is the enzyme–substrate and/or enzyme–product complex. In this model, inorganic nitrite reacts with a reduced form of XO to yield an enzyme–substrate (or enzyme–product) complex. This can break down in two ways: productively, to yield product NO plus a (relatively) oxidized form of the enzyme or destructively, to give inactivated enzyme.

Figure 4 Progress curves of urate production catalysed by XO in the presence of xanthine and inorganic nitrite

XO (10.2 nM active subunits) was incubated anaerobically with xanthine and various concentrations of sodium nitrite: 120 mM (●), 30 mM (○) and 7.5 mM (■). Values are means from three individual progress curves. Solid lines represent fits to the model shown in Scheme 1, using the parameters shown in Table 1.

Figure 5 Progress curves of NADH depletion catalysed by XO in the presence of inorganic nitrite

XO (57.8 nM active subunits) was incubated anaerobically with NADH and various concentrations of sodium nitrite: 120 mM (●), 30 mM (○) and 7.5 mM (■). Values are means from three individual progress curves. Solid lines represent fits to the model shown in Scheme 1, using the parameters shown in Table 1.

In addition, the model incorporates a step that involves the inactivation of reduced enzyme by enzyme-generated free NO in a second-order reaction, as proposed by Ichimori et al. [36]. The model is defined by the following relationships, where \( E_T \) represents total enzyme concentration:

\[ \frac{\text{d}[\text{urate}]}{\text{d} t} = k \cdot [\text{xanthine}] \]  
\[ [\text{E}_\text{R} \cdot \text{NO}_2^-] = [\text{NO}_2^-] \cdot [\text{E}_\text{X} - [\text{E}_\text{OX} - [\text{E}_\text{R}])] / (K_M + [\text{NO}_2^-]) \]  
\[ \frac{\text{d}[\text{E}_\text{X}]}{\text{d} t} = k_i [\text{E}_\text{R} \cdot \text{NO}_2^-] + k_i [\text{NO}] [\text{E}_\text{R}] \]  
\[ \frac{\text{d}[\text{E}_\text{OX}]}{\text{d} t} = k_{\text{cat}} [\text{E}_\text{R} \cdot \text{NO}_2^-] \]

Xanthine concentration was held constant at a value such that depletion of xanthine does not occur to any significant extent.
Table 1 Kinetic parameters used in the fits of the model of Scheme 1 to the progress curves shown in Figures 4, 5, 6 and 10

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$K_v$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_i$ (s$^{-1}$)</th>
<th>$k_{cat}/k_i$</th>
<th>$k_i$ (M$^{-1}$·s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XO + xanthine + NaNO$_2$</td>
<td>280±25</td>
<td>7.2±0.6</td>
<td>0.012±0.001</td>
<td>630±92</td>
<td>14.8</td>
</tr>
<tr>
<td>XO + NADH + NaNO$_2$</td>
<td>33±1.2</td>
<td>0.7±0.02</td>
<td>0.0016±0.0002</td>
<td>450±44</td>
<td>14.8</td>
</tr>
<tr>
<td>XDH + NADH + NaNO$_2$</td>
<td>14±0.1</td>
<td>2.4±0.03</td>
<td>0.0033±0.001</td>
<td>740±33</td>
<td>14.8</td>
</tr>
<tr>
<td>XO + xanthine + Na$_2$O$_4$</td>
<td>48±0.3</td>
<td>1.6±0.04</td>
<td>0.0003±0.0001</td>
<td>6100±310</td>
<td>–</td>
</tr>
</tbody>
</table>

Values of $K_v$ and $k_{cat}$ were calculated from the initial rates of the progress curves assuming Michaelis–Menten kinetics and no initial inactivation. $k_i$ is assigned the value found by Ichimori et al. [36]. Values of $k_i$ obtained by fitting the model of Scheme 1 to the progress curves. Experimental conditions are described in the legends to Figures 4, 5, 6 and 10.
The different forms of the enzyme (150 nM active subunits) were incubated under anaerobic conditions with 100 μM xanthine and various concentrations of sodium nitrate. Data for XO (●) and IoDP-inhibited XO (○) were fitted to the same Michaelis–Menten curve. Values are means ± S.D. (n = 3).

Figure 9 Inhibition by nitrate of NO production from nitrite catalysed by XO in the presence of xanthine

XO (153 nM active subunits) was incubated under anaerobic conditions with 20 μM xanthine and various concentrations of sodium nitrate and sodium nitrite. Initial rate of NO production ν is expressed in nmol·min⁻¹·mg⁻¹. (a) Dixon plot [43] of 1/ν versus concentration of inorganic nitrate at different concentrations of sodium nitrite. (b) Cornish-Bowden plot [44] of s/ν versus concentration of inorganic nitrate at different concentrations of sodium nitrite. Concentrations of sodium nitrite were 100 mM (●) and 30 mM (○). Both plots indicate predominantly competitive inhibition of nitrate against nitrite. Values are means ± S.D. (n = 3).

Figure 10 Progress curves of urate production catalysed by XO in the presence of xanthine and inorganic nitrate

XO (27.4 nM active subunits) was incubated anaerobically with xanthine and various concentrations of sodium nitrate: 60 mM (●), 15 mM (○) and 3.75 mM (▲). Values are means from three individual progress curves. Solid lines represent fits to the model shown in Scheme 1, using the parameters shown in Table 1.
to were found to fit well to the model of Scheme 1, where of XO, xanthine and inorganic nitrite under anaerobic conditions in the progress curves themselves. Mechanisms (see the Results section) and clarification was sought these means to determine the relative contributions of the two predominance of the latter route (Figure 3), it is difficult by –product complex. Whereas the kinetics of inactivation indicate which indicates strongly that the latter directly reflects the former.

320 nm, we have been able to demonstrate correlation of the rates of sulpho–desulpho conversion and inactivation (Figure 2), and inorganic nitrite and/or suicide inactivation (Figure 3), it is difficult by these means to determine the relative contributions of the two mechanisms (see the Results section) and clarification was sought in the progress curves themselves.

Progress curves (Figure 4) of urate production in the presence of XO, xanthine and inorganic nitrite under anaerobic conditions were found to fit well to the model of Scheme 1, where $k_{in}$ and $k_{out}$ are derived from the linear initial rates and $k_i$ is that quoted by Ichimori et al. [36]. Good fits were similarly obtained to progress curves in the presence of XO and NADH (Figure 5) and in the presence of XDH and NADH (Figure 6). The ratio of $k_{out}$ to $k_i$ (Table 1) can be regarded as a relative indicator of the number of productive turnovers per inactivation. Although the kinetic parameters differ for the three cases, it is noteworthy that this ratio remains reasonably independent of the nature of the reducing substrate (xanthine or NADH) or the form of the enzyme (oxidase or dehydrogenase), supporting the generality of the model. As noted in the Results section, the contribution to inactivation by free NO, based on the second-order rate constant in [36], contributed little to the overall inactivation.

The kinetic constants (Table 1) derived from fits of the model of Scheme 1 should be seen as operational parameters, rather than true rate constants. This is because the species designated $E_n$ is a composite of the various reduced enzyme molecules capable of interacting productively (or destructively) with nitrite (or NO). We have demonstrated previously the 1:2 stoichiometric ratio of xanthine [20] or NADH [18] consumption to NO production in the presence of inorganic nitrite and that the latter is subjected to one-electron reduction at the Mo site [18]. Thus $E_n$ may be regarded as a composite of enzyme species with Mo in its IV and V oxidation states, and the mechanism of reduction as a composite of routes A and B in Scheme 2, where E–Mo$^{IV}$ and E–Mo$^{V}$ are those $E_n$ species (Scheme 1) actively interacting with nitrite and $E_n$ is the inactivated desulpho-form of the enzyme.

Inorganic nitrate has been shown to be reduced to NO in the presence of NADH and XO under hypoxic conditions [46]. In the present study, we show that inorganic nitrate, like nitrite [18], is reduced at the molybdenum enzyme site of the enzyme. This, incidentally, is in contrast to organic nitrates, which act at the FAD site [47]. We also show that, in the presence of xanthine and nitrate, XO catalyses urate and nitrite production in essentially a 1:1 ratio; nitrite is presumably further reduced to NO at relatively insignificant rates in this system. The reason for such low rates of NO production may be at least partially explained in terms of competition between nitrate and nitrite for reduced enzyme.

XOR-catalysed reduction of nitrate, like that of nitrite, might be expected to be initiated by nucleophilic displacement of -SH or -OH from reduced molybdenum (see above). In so far as this displacement contributes to enzyme inactivation, it would apply equally to nitrate and nitrite. On the other hand, inactivation at a later stage, involving an intermediate enzyme–NO complex, applies primarily to reduction of nitrite, in that nitrate generates very low levels of NO and only after initial nitrate formation. In fact, incubations with nitrate showed little evidence of inactivation, as shown by both discontinuous assay and progress curves. The relative absence of enzyme inactivation with nitrate indicates that nucleophilic displacement of -SH is not occurring. This strongly suggests that a similar situation prevails in the case

![Scheme 2 Postulated involvement of various Mo redox states of XOR in the reduction of inorganic nitrite and/or suicide inactivation](image)

E–Mo$^{IV}$ and E–Mo$^{V}$ are those $E_n$ species (Scheme 1) actively interacting with nitrite and $E_n$ is the inactivated desulpho-form of the enzyme.
of nitrite and that inactivation results from subsequent interaction of nascent NO with molybdenum-bound sulphur in the enzyme complex.

These conclusions do not contradict the findings of Ichimori et al. [36], who were studying an entirely different situation, involving inactivation of pre-reduced enzyme by relatively high concentrations of added NO. In fact, we cannot discount involvement of an analogous inactivation pathway in our system. The concentrations of free NO and consequent rates of inactivation are merely too low to make a major contribution in the course of XOR-catalysed reduction of inorganic nitrite. Indeed, our conclusion that inactivation occurs primarily at the stage of the enzyme–nitrite–NO complex can be seen as consistent with the findings of Ichimori et al. [36], in so far as NO, in a free or complexed form, can interact with sulphur bound to a reduced form of molybdenum to give the desulpho-form of the enzyme complex.

Inactivation of XOR in the course of nitrite reduction in the mammary gland may well serve to explain the presence of desulpho-XOR in freshly expressed milk, whether of bovine or human origin [2, 7, 11]. We have noted previously that XOR-catalysed generation of NO could serve a physiological function as a microbicidal agent in the neonatal gut [18, 19] and similar arguments apply to the mammary gland. Supporting evidence for such a role in either location is provided by the observations that levels of XOR enzyme activity [48, 49] and levels of nitrite [50] are particularly high in early post-partum breast milk; factors that would combine to maximize microbicidal activity.

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