Reduction of Nitrite to Nitric Oxide Catalyzed by Xanthine Oxidoreductase*

(Received for publication, September 16, 1999, and in revised form, December 16, 1999)

Ben L. J. Godber‡, Justin J. Doel‡‡, Gopal P. Sapkota‡, David R. Blake‡, Cliff R. Stevens‡, Robert Eisenthal‡, and Roger Harrison‡‡

From the Departments of ‡Biological Sciences and §Postgraduate Medicine, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom

Xanthine oxidase (XO) was shown to catalyze the reduction of nitrite to nitric oxide (NO), under anaerobic conditions, in the presence of either NADH or xanthine as reducing substrate. NO production was directly demonstrated by ozone chemiluminescence and showed stoichiometry of approximately 2:1 versus NADH depletion. With xanthine as reducing substrate, the kinetics of NO production were complicated by enzyme inactivation, resulting from NO-induced conversion of XO to its relatively inactive desulfo-form. Steady-state kinetic parameters were determined spectrophotometrically for urate production and NADH oxidation catalyzed by XO and xanthine dehydrogenase in the presence of nitrite under anaerobic conditions. pH optima for anaerobic NO production catalyzed by XO in the presence of nitrite were 7.0 for NADH and ≤6.0 for xanthine. Involvement of the molybdenum site of XO in nitrite reduction was shown by the fact that alloxahtine inhibits xanthine oxidase competitively with nitrite. Strong preference for Mo=S over Mo=O was shown by the relatively very low NADH-nitrite reductase activity shown by desulfo-enzyme. The FAD site of XO was shown not to influence nitrite reduction in the presence of xanthine, although it was clearly involved when NADH was the reducing substrate. Apparent production of NO decreased with increasing oxygen tensions, consistent with reaction of NO with XO-generated superoxide. It is proposed that XO-derived NO fulfills a bactericidal role in the digestive tract.

Xanthine oxidoreductase (XOR) is a complex flavoprotein comprising two identical subunits of M₉ 145,000. Each subunit contains one molybdenum, one FAD, and two nonidentical iron-sulfur redox centers (1, 2). Although it has broad specificities for both reducing and oxidizing substrates, its conventionally accepted role is in purine catabolism, where it catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid. In mammals, XOR exists in two interconvertible forms, xanthine dehydrogenase (XDH; EC 1.1.1.204), which predominates in vivo, and xanthine oxidase (XO; EC 1.1.3.22). Both forms of the enzyme can reduce molecular oxygen, although only XDH can reduce NAD, which is its preferred electron acceptor. Reduction of oxygen leads to superoxide anion and hydrogen peroxide; it is the potential to generate these reactive oxygen species that has led to widespread interest in the enzyme as a pathogenic agent in many forms of ischemia-reperfusion injury (3). More recently, reactive oxygen species are increasingly cited as intermediates in normal signal transduction pathways (4, 5).

Nitric oxide (NO) also is a biological messenger and, over the last decade, has become the focus of intense research activity in its own right. It is implicated in a wide range of physiological processes, including smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission, and immune regulation (6–9). It is generally accepted that the enzymic source of NO in mammalian tissues is NO synthase, which catalyzes the oxidation of arginine in the presence of NADPH and molecular oxygen (10). We have recently briefly reported that XO is capable of catalyzing the reduction of glycercyl trinitrite, as well as inorganic nitrate and nitrite, to NO under hypoxic conditions in the presence of NADH (11). We now examine the kinetics and stoichiometry of NO production catalyzed by XOR in the presence of inorganic nitrite and both NADH and xanthine as reducing substrates under conditions of different oxygen tensions and provide evidence for the sites of action of the various substrates.

EXPERIMENTAL PROCEDURES

Xanthine Oxidase and Xanthine Dehydrogenase—XOR was purified from bovine milk, essentially as described by Sanders et al. (12) with minor modifications. Fresh milk (1500–2000 ml) was centrifuged (2000 × g, 30 min) at 4 °C to separate the cream. All subsequent operations were carried out at 4 °C. The cream was resuspended in 5 volumes of 0.2 M K₂HPO₄, containing 1 mM EDTA and 5.0 mM dithiothreitol, with stirring for 90 min. The suspension was centrifuged (3000 × g, 30 min), and the supernatant was filtered, then centrifuged (1500 × g, 20 min) and filtration of the supernatant through glass wool, and mixed slowly with 15% (by volume) cold butanol (–20 °C). Ammonium sulfate (15 g/100 ml) was added without stirring, which was continued for a further 50 min, before centrifugation (8000 × g, 20 min) and filtration of the supernatant through glass wool. Ammonium sulfate (20 g/100 ml) was then added slowly to the supernatant with mixing, and the suspension was stirred for a further 30 min before centrifuging (9500 × g, 30 min). The brown precipitate was suspended in a small volume of buffer A (25 mM Na/MES, pH 6.0, containing 1 mM EDTA) and dialyzed against the same buffer (3.5 liters) overnight. Any precipitate was removed by centrifugation (10,000 × g, 60 min) and the supernatant was filtered through a 0.22-μm membrane, before applying to a column (1 × 9 cm) of heparin immobilized on cross-linked 4% beaded agarose (Sigma, type 1), previously equilibrated in buffer A. The column was washed with 50 mM NaCl in buffer A (20 ml), and XOR was eluted with 0.3 M NaCl in the same buffer.

Further purification was effected by ion exchange chromatography.
Eluate from the heparin column was dialyzed into 50 mM Na/Bicine, pH 8.3, containing 50 mM NaCl, filtered through a 0.22-μm syringe filter, and applied to an equilibrated HiTrap Q ion exchange column (Amer sham Pharmacia Biotech) for fast protein liquid chromatography. The column was eluted with 50 mM Na/Bicine, pH 8.3, followed by an increased salt gradient (0.65–1.0 M NaCl) in buffer, when enzyme-containing fractions were pooled and concentrated.

Typical yields after heparin chromatography were 15 mg of enzyme protein/liter of milk, with A450/A250 5.0–5.5. Mono Q chromatography yielded enzyme with A280/A340 ratios of 5.0–5.2 in yields of approximately 85%. The enzyme-containing eluate from HiTrap Q was dialyzed overnight into 50 mM Na/Bicine, pH 8.3, and frozen dropwise in liquid nitrogen, and stored at −80 °C until required.

Following thawing, the enzyme showed >97% oxidative activity as determined by the method described below and constitututed XO for the studies described here. XDH was obtained by incubation of purified XO 50 mM Na/Bicine, pH 8.3, containing 10 mM dithiothreitol for 40 min at 37 °C. Dithiothreitol was subsequently removed by gel filtration on Sephadex G25. XDH prepared by these means contained >98% dehydrogenase form.

The oxidase content of XOR was 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 mM−1 cm−1 (13). Assays were performed at 25.0 ± 0.2 °C in air-saturated buffer, pH 8.3, containing 100 μM xanthine. The sum of oxidative and dehydrogenase contents was determined as above but in the presence of 0.5 mM NAD.

The enzyme used contained 35–38% functional active sites, as judged by the activity:flavin ratio, assuming fully active enzyme has an activity:flavin ratio of 208 (14). Concentrations of enzyme were determined from the UV-visible spectrum, by using an absorption coefficient of 36 10340 nm using an absorption coefficient of 6.22 mM−1 cm−1 (19).

**RESULTS**

**Stoichiometry of NO Production and Enzyme Inactivation under Anaerobic Conditions—**XO-catalyzed generation of NO in the presence of nitrite could be directly demonstrated by ozone chemiluminescence (see "Experimental Procedures"). In the presence of NADH, rates of NO production and NADH depletion both followed Michaelis-Menten kinetics (Fig. 1), showing a stoichiometry (see "Experimental Procedures") of (2.19 ± 0.15):1, consistent with transfer from NADH of pairs of electrons (12), each of which reduces one molecule of nitrite to NO.

With xanthine as reducing substrate, the stoichiometry of NO versus urate production was considerably less than 2:1, approximating rather 1:1 (data not shown). In view of reports that XO is inactivated by NO (20–22), particularly in the presence of xanthine (22), we sought evidence for inactivation under the experimental conditions of NO generation. As can be seen in Fig. 2, in the presence of xanthine the enzyme is indeed progressively inactivated over the time course of NO production. After 2 min, the point at which the NO reading is commonly taken (see "Experimental Procedures"), inactivation has reached approximately 60%, a value consistent with the

**FIG. 1. Stoichiometry of anaerobic NO production catalyzed by XO in the presence of NADH and nitrite.** XO (0.2 μM) was incubated in nitrogen-sparged 50 mM potassium phosphate buffer, pH 7.2, at 37 °C ± 0.2 °C with 100 μM NADH and varying concentrations of sodium nitrite. NO production (●) was determined by using a chemiluminescence NO detector (see "Experimental Procedures"). NADH depletion (○) was followed by monitoring absorbance at 340 nm (see "Experimental Procedures") in an anaerobic cabinet (<3 ppm O2). Michaelis-Menten curves were fitted to the data. Stoichiometry of NO production versus NADH depletion was calculated from the respective Vmax values. Values are means ± S.D. (n = 3). Where error bars are not shown, their width is less than that of the symbol.
yield 90% inactivated enzyme (450 nM xanthine, as outlined in the legend to Fig. 2. The reaction mixture was taken at different time intervals and assayed for urate production by monitoring absorbance at 295 nm (see "Experimental Procedures"). The percentage of initial activity is plotted against time.

Approach to 1:1 stoichiometry discussed above.

Ichimori et al. (22) demonstrated inactivation of conventional aerobic XO activity by exogenous NO and attributed this inactivation to generation of desulfo-XO, in which MoS3 is replaced by Mo=O. In the present context, it was clearly of interest to determine whether inactivation observed in the course of NO generation results from desulfuration of XO. A sample of XO (initial activity of 4500 ± 120 nmol of urate min⁻¹ mg⁻¹) was incubated anaerobically for 20 min in the presence of nitrite and xanthine, as outlined in the legend to Fig. 2. The reaction mixture was gel-filtered (see "Experimental Procedures") to yield 90% inactivated enzyme (450 ± 30 nmol of urate min⁻¹ mg⁻¹), which could, indeed, be reactivated on sulfuration (see "Experimental Procedures") (final activity of 5430 ± 290 nmol of urate min⁻¹ mg⁻¹). This is consistent with the conclusion that NO, whether exogenously added (22) or endogenously generated in the presence of xanthine, inactivates XO by transforming it to the desulfo-form. In contrast to the situation observed with xanthine, rates of enzyme inactivation were very much lower in the presence of NADH, leading to less than 5% inactivation after 2 min (Fig. 2).

**Steady-state Kinetics of the Reduction of Nitrate by NADH or Xanthine Catalyzed by XO or XDH under Anaerobic Conditions**—The kinetics of the reduction of nitrate by NADH catalyzed by XO were followed by observing the rate of NADH depletion at 340 nm. Treating either nitrate or NADH as the varied substrate, the variation of rate with substrate concentration follows Michaelis-Menten kinetics. With xanthine as reducing substrate, the reaction rates were monitored by following urate accumulation at 295 nm. When nitrite is treated as the variable substrate concentration, Michaelis-Menten behavior was again found. However, the variation of rate with xanthine concentration fitted well to Equation 1 for substrate inhibition (Fig. 3), where \( K_s \) is the Michaelis constant and \( K_i \) is the substrate inhibition constant.

\[
\frac{v}{V_{\text{max}}} = \frac{V_{\text{max}}}{1 + K_s[S] + [S]/K_i}
\]  

Substrate inhibition was relieved by increasing nitrate concentration; over the range of 20–120 mM nitrate, \( K_i \) varied from 30 to 239 \( \mu \)M.

Using XDH, with xanthine as the reducing substrate, the kinetic behavior was identical to that using XO (Fig. 4). However, with NADH as the reducing substrate, Michaelis-Menten kinetics were followed (as with the oxidase), but the parameters were significantly different from those obtained using the oxidase form (Table I).

Because of the substrate inhibition by xanthine, it was not possible to draw any mechanistic conclusions from the values of the apparent steady-state kinetic parameters. However, with NADH as reducing substrate, \( V_{\text{max}}/V_m \) for XO was constant throughout the range of substrate concentrations employed, consistent with a ping-pong mechanism. Interestingly, with XDH, \( V_{\text{max}}/V_m \) increased with increasing substrate concentration for both NADH and nitrate.

All of the above kinetic experiments were carried out with enzyme of 35–38% functionality (see "Experimental Procedures"). NADH-nitrite reductase activity of desulfo-XO (see "Experimental Procedures") was low but significant, contributing rates of less than 3% in those of the standard enzyme preparation.

**pH Profiles of Anaerobic NO Production, NADH Oxidation, and Xanthine Oxidation**—The pH profiles for NO production in the presence of nitrate and NADH or xanthine catalyzed by XO under anaerobic conditions are shown in Fig. 5. Essentially
identical profiles were obtained when NADH depletion or urate production, respectively, were monitored in the presence of nitrite (data not shown).

**Involvement of Molybdenum and FAD Sites in the Catalytic Activity of XO**—Direct involvement of the molybdenum site in nitrite reduction was demonstrated by inhibition of anaerobic NADH oxidation in the presence of alloxanidine. The well-known inhibitor, allopurinol, exerts its effect by acting as a reducing substrate for XOR, thereby being itself oxidized to alloxanidine (also known as oxypurinol), which then binds very tightly to the reduced form of the enzyme. In the course of its aerobic reduction, nitrite might be expected to bind also to the reduced form of molybdenum; that this is indeed the case is indicated by the demonstration that alloxanidine inhibited NADH depletion competitively with nitrite (Fig. 6).

It was shown, furthermore, that reduction of nitrite to NO is dependent on the sulfini-form of XO. Conversion of XO to its desulfini-form (see "Experimental Procedures") resulted in apparent loss of its ability to catalyze either aerobic xanthine oxidation or anaerobic NO production in the presence of NADH and nitrite. Both of these activities were recovered, with essentially identical time courses, upon resulfuration (see "Experimental Procedures") (Fig. 7).

Urate production in the presence of xanthine and nitrite catalyzed by XO anaerobically was essentially identical to that catalyzed aerobically by IDP-inhibited XO (Fig. 8), consistent with a lack of involvement of the FAD site in nitrite reduction. With deflavo-enzyme, under aerobic conditions, rates of urate production were somewhat lower (Fig. 8), probably reflecting instability of this form of the enzyme (see "Discussion").

The presence of NADH and nitrite, neither deflavo- nor IDP-inhibited enzyme (see "Experimental Procedures") showed detectable NO production or NADH depletion (data not shown), a result that is unsurprising, in view of the well-established direct interaction of NADH with the FAD site of XO (17).

**XO-catalyzed Generation of NO in the Presence of Oxygen**—In the presence of NADH and nitrite, XO-catalyzed NO production apparently decreased as oxygen concentration increased, remaining detectable only at higher concentrations of nitrite (Fig. 9). This phenomenon can be largely attributed to rapid reaction of NO with superoxide to form peroxynitrite (27) as evidenced by the effects of superoxide dismutase. In the presence of 200 units of superoxide dismutase, the rate profile of NO production versus nitrite concentration was identical to that obtained anaerobically in the absence of superoxide dismutase (Fig. 10).

**DISCUSSION**

It has been known for over 75 years that XO has the ability to catalyze the reduction of nitrate to nitrite under anaerobic conditions (28–31). However, XO-catalyzed further reduction of nitrite to NO has only very recently been reported. We briefly described this phenomenon in the presence of NADH under hypoxic conditions (11); a subsequent communication (32) reported NO generation in both hypoxia and ambient air. The present study examines the kinetics and mechanism of nitrite reduction catalyzed by XO with both NADH and xanthine as reducing substrates and addresses the effects of oxygen.

Anaerobic NO production catalyzed by XO in the presence of nitrite and NADH could be directly demonstrated by ozone chemiluminescence using a chemiluminescence NO detector. Comparison with rates of NADH utilization showed a stoichiometric ratio of approximately 2:1 versus NO production. This result is predicted on the basis of a redox reaction comprising a two-electron oxidation of NADH to NAD⁺ and a one-electron reduction of nitrite to NO. Involving as it does both spectrophotometric and chemiluminescence NO detector data, this finding serves to validate the latter. Corresponding experiments in the presence of xanthine, rather than NADH, showed relatively less NO production. This initially surprising finding was satisfactorily explained in terms of NO-induced inactivation of XO under conditions of the assay. While it is not clear why such inactivation should be markedly greater in the presence of xanthine rather than NADH, this may reflect the extent to which the enzyme is present in reduced states (22). Our findings complement those recently reported by Ichimori et al. (32) involving inactivation of xanthine-reduced XO by exogenous NO. These authors demonstrated that enzyme inactivation in their system resulted from sulfo-desulfo conversion. We now show not only that XO can be inactivated by endogenously generated NO, but that in this case also the mechanism involves Mo=S to Mo=O conversion.

From the above discussion, it is clear that kinetic experiments in the chemiluminescence NO detector will be more or less subject to complications resulting from NO-induced inactivation of XO. This is because, as a consequence of phase
equilibration and gas flow factors, data in our system are first obtained approximately 2 min after initiation of the reaction. We accordingly preferred spectrophotometric monitoring of NADH depletion or of urate production for determination of steady-state kinetic parameters. Determination of the steady state rate can be made earlier in the time course, where product inactivation is less of a consideration, and this allowed determination of the kinetic parameters shown in Table I.

The substrate steady-state kinetics are not intended to provide mechanistic information as such but rather serve to supply operational parameters with which to estimate the rates of XO-catalyzed NO production that might be expected to occur in vivo (see below). However, one point deserves comment. XDH is the predominant form (34). Inhibition of NADH oxidation by alloxanthine competes more efficiently with respect to nitrite implicates molybdenum as the site of nitrite reduction and is fully consistent with interaction of nitrite with the reduced form of XO. We have shown, moreover, that NO production in the presence of nitrite and NADH depletion is initially examined by monitoring absorbance at 340 nm. Initial rate (v) is expressed in nmol min$^{-1}$, and Mo(V)/Mo(IV) (300 mV) and Mo(V)/Mo(III) (−300 mV). The corresponding values for desulfo-enzyme are quoted as −335 and −309 mV. In the context of midpoint potentials for FAD/FADH$^2$ and FADH/FADH$^2$, of −235 and −215 mV, respectively (35), transfer of electrons from NADH to molybdenum via the FAD site will be less favorable in the case of desulfo-enzyme. Possible influence of the FAD center of XO on xanthine oxidation in the presence of nitrite was initially examined by comparing XO and deflavo-XO. While rates of urate production

**Fig. 6.** Inhibition by alloxanthine of anaerobic NADH oxidation catalyzed by XO in the presence of nitrite. XO (360 nM) in nitrogen-sparged potassium phosphate buffer, pH 7.2, was incubated at 37 ± 0.2 °C in an anaerobic cabinet (<3 ppm O$_2$) with 100 μM NADH with varying concentrations of sodium nitrite and alloxanthine. NADH depletion was followed by monitoring absorbance at 340 nm. Initial rate (v) is expressed in nmol min$^{-1}$, ml$^{-1}$. A, Dixon plot (25) of 1/v versus concentration of alloxanthine at different concentrations of nitrite. Concentrations of nitrite are 10 mm (○), 20 mm (□), and 40 mm (×). Both plots are diagnostic for simple competitive inhibition of alloxanthine against nitrite. Values are means ± S.D. (n = 3).

**Fig. 7.** Reactivation of CN-treated XO. XO (initial activities 4000 ± 200 nmol of urate min$^{-1}$ mg$^{-1}$ and 40.5 ± 2.3 nmol of NO min$^{-1}$ mg$^{-1}$) was converted to its desulfo-form by treatment with cyanide. It was then resulfurated under anaerobic conditions, during which period aliquots were collected and gel-filtered and assayed for enzyme activity (see “Experimental Procedures”). Urate production (●) was determined aerobically in the presence of 100 μM xanthine. NO generation (□) was measured anaerobically in the presence of 100 μM nitrite and 500 μM NADH. Values are means ± S.D. (n = 3).

**Fig. 8.** Xanthine oxidation in the presence of nitrite catalyzed by XO, IDP-inhibited XO, or deflavo-XO. The different forms of the enzyme were incubated with 100 μM xanthine at 37 ± 0.2 °C in potassium phosphate buffer, pH 7.2, and varying concentrations of sodium nitrite. Experiments with XO were carried out in nitrogen-sparged buffer in an anaerobic cabinet (<3 ppm O$_2$). Those using IDP-inhibited XO and deflavo-XO (see "Experimental Procedures") were done in air-saturated buffer. In all cases, urate production was monitored at 285 nm. Data for XO (●) and IDP-inhibited XO (□) were fitted to the same Michaelis-Menten curve. Those for deflavo-XO (×) were fitted to a different Michaelis-Menten curve. Values are means ± S.D. (n = 3).
Nitric Oxide from Xanthine Oxidoreductase

were similar, those with deflavo-enzyme were slightly lower, an observation that might be attributable to instability of this enzyme form, which is prone to aggregation and precipitation (17). Accordingly, use was made of IDP, which, like diphenylene iodonium (18), was shown to inactivate the FAD site of XO, as judged by loss of xanthine oxidase activity (see “Experimental Procedures”). In this case, rates of urate production catalyzed by XO and flavin-inactivated enzyme were essentially identical, confirming the lack of influence of the FAD site in this reaction. In contrast, NADH depletion in the presence of nitrite was totally inhibited by IDP.

The above experiments concerning the mechanisms of NO production were carried out with XO. The similarity in kinetic behavior of XO and XDH in catalyzing the reaction between nitrite and xanthine (Fig. 4), suggests that conclusions drawn on the basis of XO may also be applicable to XDH.

In the presence of oxygen, the situation is complicated by concomitant generation of NO and superoxide anion. As oxygen tensions rise, levels of NO production were seen to fall, becoming undetectable at lower concentrations of nitrite. These effects of oxygen could be completely reversed by the addition of superoxide dismutase, indicating that they can be entirely accounted for by rapid reaction of NO with superoxide, presumably yielding peroxynitrite (36). Rates of superoxide production in the presence of NADH were shown to correspond closely to the observed decrease in detected NO as oxygen tensions rise (data not shown).

An obvious issue concerns the physiological role of XOR-catalyzed generation of NO. Indeed, the enzyme’s function in vivo has long been debated, particularly as its rather specialized location in endothelial, epithelial, and connective tissue cells implies more than a housekeeping role in purine catabolism (37). In the context of vascular endothelium, the capacity of XOR to produce superoxide and hydrogen peroxide has led to its implication in the inflammatory process (38, 39). More recently, attention has focused on interactions of superoxide with endogenous NO and their possible deleterious consequences in atherosclerosis (40–42).

Our finding that XOR itself can also catalyze NO production adds a new dimension to these discussions. XOR can be seen as complementary to NO synthase, in that, unlike the latter (10), it is capable of producing NO under anoxic conditions. XOR could, accordingly, promote NO-induced vasodilation in ischemia, when NO synthase activity is low. NO synthase is induced under such conditions (43), prior to assuming the burden of NO production as oxygen tension rises.

In view of our present observations, the subcellular localization of the enzyme assumes particular importance. Until relatively recently, XOR has been generally assumed to be exclusively cytoplasmic (44), where XDH predominates (34). In this context, XDH would certainly be more efficient than XO as a catalyst of NO production, at least with NADH as reducing substrate (Table I). Of late, attention has been directed to extracellular endothelial XOR, arising either endogenously (45, 46) or via the circulation. In the presence of serum, the oxidase form, XO, predominates (34). Levels of circulating enzyme are normally low (47) but can rise dramatically in a variety of inflammatory disease states, particularly those involving liver damage (48–50). XO has been shown to bind to vascular endothelium (41) by mechanisms that probably involve specific interactions with glycosaminoglycans (51, 52), and the enzyme may be expected to be greatly concentrated at the luminal surface. In this context, superoxide dismutase assumes relevance. Mammalian extracellular superoxide dismutase C has strong affinity for heparin (53), and its immediate colocalization with XO could conceivably result in similar rates of NO production in the presence or absence of oxygen (Fig. 10).

In the absence of significant interactions with superoxide dismutase, XO is a potential source of peroxynitrite, with its capacity for endothelial injury (54). Indeed, levels of both XO (55) and anti-XO antibodies (56) have been shown to be elevated in plasma of atherosclerosis patients, and the enzyme has been detected in their vessel walls and plaques (57, 58).

Maximal rates of XOR-catalyzed nitrite reduction (Table I) are significant in the context of estimated rates of NO production in the vasculature, which, according to Beckman et al. (59), range up to 8 μM min⁻¹. However, Km values for nitrite are in the micromolar range (Table I), considerably higher than the micromolar levels to be found in plasma (60). This prompts consideration of the enzyme’s role in other physiological environments. In particular, XO could fulfill a bactericidal function in the digestive tract, throughout which it is present in epithelial cells (61). By way of the milk fat globule membrane (62), it
Nitric Oxide from Xanthine Oxidoreductase

also occurs in the neonatal gut. While levels of nitrate in the digestive tract are generally in the micromolar range (63, 64), they are potentially much higher in the microenvironment of enteric bacteria, which, at least in anaerobic culture, can excrete millimolar levels of nitrite (65, 66), derived from dissipative nitrate reductase (67). Under such conditions and in the presence of micromolar levels of purines, XOR can generate NO, a potent bactericidal agent (68–70), at rates of approximately 1000 nmol min$^{-1}$ g$^{-1}$ tissue (71). This would yield rates of NO production of about 100 pmol min$^{-1}$ g$^{-1}$ tissue, a value that can be seen as significant in the context of rates of NO generation by activated macrophages (1 nmol min$^{-1}$ g$^{-1}$ macrophage protein (72)). It is noteworthy that the pH optimum for anaerobic XO-activated NO production in the presence of xanthine is pH 6 or less (Fig. 5), much lower than the value (pH 8.8) observed for aerobic xanthine oxidase activity (73) and more appropriate to a role in the digestive tract.

As oxygen tensions rise, so does the potential for the generation of peroxynitrite, also a powerful bactericide (74). It is an attractive concept that, by way of nitrite excretion, enteric bacteria might initiate their own destruction, a concept strengthened by the known affinity of XOR for acidic polysaccharides (51, 52), such as occur in many bacterial capsules (75). While much of the above argument is clearly speculative, it is supported by recent ultrastructural studies (61) in the rat intestinal epithelial cells showed signs of destruction and/or death and were surrounded by XOR.

In summary, we have shown that, in the presence of NADH or xanthine as reducing substrate, XOR can catalyze the reduction of nitrite to NO. Nitrite reduction takes place at the molybdenum site, and is dependent upon the presence of the Mo=O$^\cdot$ group, and, in the presence of xanthine, is unaffected by loss of activity at the FAD site. As oxygen tension increases, the yield of NO decreases as a consequence of its reaction with XOR-generated superoxide. XOR-catalyzed production of NO is potentially important in signaling events in vascular endothelium and as a bactericidal system in the digestive tract.

Acknowledgments—Andrew Downes is gratefully acknowledged for preparation of enzyme. We also thank Dr. Tim Millar (University of Bath), Professor Jeff Cole (Birmingham University), and Professor David Lloyd (Cardiff University) for helpful discussions.

REFERENCES

1. Bray, R. C. (1975) in The Enzymes (Boyer, P. D., ed) Vol. 12, 3rd Ed., pp. 299–420, Academic Press, New York.
2. Bray, R. C. (1988) Q. Rev. Biophys. 21, 299–329
3. McCord, J. M. (1975) in The Enzymes (Boyer, P. D., ed) Vol. 12, 3rd Ed., pp. 315–363, Academic Press, New York.
4. Khan, A. U., and Wilson, T. (1995) Biochem. J. 312, 159–163
5. Winograd, P. G., and Bray, R. C. (1988) Adv. Pharmacol. 38, 403–421
6. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 43–142
7. Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992) Science 258, 1898–1902
8. Stamler, J. S. (1994) Cell 78, 931–936
9. Farrell, A. J., and Blake, D. R. (1996) Ann. Rev. Physiol. 57, 7–20
10. Knowles, R. G., and Moncada, S. (1994) Biochem. J. 298, 249–258
11. Millar, T. M., Stevens, C. R., Benjamin, N., Eisenhal, R., Harrison, R., and Blake, D. R. (1997) FEBS Lett. 427, 225–230
12. Sanders, S. A., Eisenhal, R., and Harrison, R. (1997) Eur. J. Biochem. 249, 541–548
13. Avis, P. G., Bergel, F., and Bray, R. C. (1956) J. Chem. Soc. 1219–1228
14. Moyle, V., Komai, H., Palmer, G., and Elion, G. B. (1970) J. Biol. Chem. 245, 2837–2844
15. Massey, V., and Edmondson, D. (1970) J. Biol. Chem. 245, 6595–6598
16. Wahl, R. C., and Rajagopalan, K. V. (1982) J. Biol. Chem. 257, 1354–1359
17. Komai, H., Massey, V., and Palmer, G. (1969) J. Biol. Chem. 244, 1692–1700
18. O’Donnell, R. V., Smith, G. C. M., and Jones, O. T. G. (1994) Mol. Pharmacol. 46, 778–785
19. Hessele, G., and Kornberg, A. (1948) J. Biol. Chem. 175, 385–390
20. Hassoun, P. M., Yu, F. S., Zuluta, J. J., White, A. C., and Lanzillo, J. J. (1995) Am. J. Physiol. 268, L809–L817
21. Cote, C. G., Yu, F. S., Zuluta, J. J., Vosatka, R. J., and Hassoun, P. M. (1996) Am. J. Physiol. 271, L869–L874
