A new route to peroxynitrite: a role for xanthine oxidoreductase

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Abstract Peroxynitrite, a potent oxidising, nitrating and hydroxylating agent, results from the reaction of nitric oxide with superoxide. We show that peroxynitrite can be produced by the action of a single enzyme, xanthine oxidoreductase (XOR), in the presence of inorganic nitrite, molecular oxygen and a reducing agent, such as pterin. The effects of oxygen concentration on peroxynitrite production have been examined. The physiologically predominant dehydrogenase form of the enzyme is more effective than the oxidase form under aerobic conditions. It is proposed that XOR-derived peroxynitrite fulfils a bactericidal role in milk and in the digestive tract.

Key words: Xanthine oxidase; Peroxynitrite; Nitrite; Superoxide; Nitric oxide; Xanthine dehydrogenase

1. Introduction

Peroxynitrite is a potent oxidising, nitrating and hydroxylating agent, that has attracted increasing scientific interest over the last decade [1–3]. It can mediate the oxidation of a range of biological molecules, including sulphydryl compounds, lipids, proteins and DNA [4–8]. Although the precise mechanisms may differ, this species is powerfully cytotoxic [9] and is believed to contribute to tissue injury in a range of human diseases [10]. A major source of peroxynitrite is the diffusion-limited reaction of nitric oxide (NO) with superoxide anion [11]. Superoxide anion can occur as a consequence of the activities of several enzymes, including mitochondrial oxidases, xanthine oxidoreductase (XOR), or any one of various NAD(P)H oxidases [12,13]. NO, on the other hand, is generally assumed to arise solely via NO synthase-catalysed oxidation of arginine in the presence of NADPH and molecular oxygen [14]. However, we have recently described an entirely new route to NO, involving reduction of nitrates or nitrites, catalysed by XOR [15–17].

XOR is a molybdoflavoprotein [18], existing as a homodimer, each 147 kDa subunit of which contains one molybdenum atom, one FAD and two Fe_2S_2 centres [18,19]. The enzyme has a wide specificity for reducing substrates, although its conventionally accepted role is in purine catabolism, where it catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid. In mammals, XOR occurs 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, and 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 ischaemia–reperfusion injury [20].

We initially reported that XO catalyses the reduction of the nitrovasodilator, glyceryl trinitrate, inorganic nitrate and inorganic nitrite to NO in the presence of NADH under hypoxic conditions [15]. Subsequently, we have investigated the reactions involving inorganic nitrite in greater detail, deriving steady-state kinetic parameters for XOR-catalysed generation of NO, under anaerobic conditions, in the presence of either NADH or xanthine as reducing substrate [16]. In the course of these latter studies, we observed that, in the case of XO, apparent production of NO decreased with increasing oxygen tensions, consistent with reaction of NO with enzyme-generated superoxide. We now report the direct determination of peroxynitrite generated as a result of these XOR-catalysed reactions and show that XDH is much more effective than XO as a source of peroxynitrite under aerobic conditions.

2. Materials and methods

2.1. Reagents

Dithiothreitol was purchased from the Alexis Corp. Oxygen-free nitrogen (< 50 ppm O_2) and compressed air were from British Oxygen Corporation. SIN-1 was purchased from Calbiochem reagents. Superoxide dismutase (SOD) (bovine erythrocyte) and all other reagents, unless otherwise stated, were from Sigma–Aldrich Corp. Stock solutions of dihydrorhodamine 123 (DHR) were made up to 10 mM in acetonitrile and stored at −20°C in air-tight containers protected from light.

2.2. XOR

XO was purified from bovine milk as described by Godber et al. [16], in yields of approximately 15 mg/l of fresh milk. Purified enzyme was dialysed overnight into 50 mM Na/Bicine, pH 8.3, frozen dropwise in liquid nitrogen and stored at −70°C until required. The enzyme showed A_{280}/A_{450} 5.0–5.2 and, following thawing, contained $> 97\%$ oxidase activity, as determined by the method described below. It contained 35–38% functional active sites, as judged by activity to flavin ratio (AFR), assuming fully active enzyme has an AFR of 208 [21].

XDH was prepared by incubating XO in the presence of 10 mM dithiothreitol at 37°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 mM^{-1} subunit cm^{-1} at 450 nm [18].

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Abbreviations: XOR, xanthine oxidoreductase; XO, xanthine oxidase; XDH, xanthine dehydrogenase; NO, nitric oxide; DHR, dihydrorhodamine 123; IDP, iodonium diphenyl; SOD, superoxide dismutase

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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 mM^{-1} cm^{-1} [22]. 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^+.

2.3. Determination of NO

NO generation was followed by using an ozone chemiluminescence assay in a continuous flow apparatus (Sievers NOA 280) as described by Godber et al. [16]. Reactions were carried out at 25.0 ± 0.2°C in 50 mM potassium phosphate buffer, pH 7.2, containing enzyme, sodium nitrite and pterin.

That detection of NO was independent of oxygen tension was shown by the following control experiment. IDP-inhibited XO, which catalyses production of NO but not superoxide (see [16] and Section 3), was incubated with nitrite and a reducing substrate. Rates of NO production were found not to vary significantly as oxygen concentrations varied between 0 and 240 μM.

2.4. Determination of peroxynitrite

Peroxynitrite production was determined by monitoring the oxidation of 100 μM DHR to rhodamine spectrophotometrically at 500 nm using an absorption coefficient of 78.8 M^{-1} cm^{-1} [23]. Assays were performed at 25.0 ± 0.2°C in 50 mM potassium phosphate buffer, pH 7.2, containing pterin and sodium nitrite in a stoppered, stirred 1 ml cuvette. Gas of defined oxygen concentration was bubbled through the solution at a depth of 1 mm and a rate of 100 ml/min. Reactions were started by addition of pterin and background rates obtained in the presence of nitrite and iodonium diphenyl (IDP, see Section 3) were subtracted from the observed rates.

Crow [23] has demonstrated the specificity for peroxynitrite of the DHR assay, which is unaffected by NO, superoxide or hydrogen peroxide.

The effect of molecular oxygen on the DHR assay was determined as follows. Peroxynitrite was generated from the reaction of hydrogen peroxide with nitrous acid and quantified spectrophotometrically as described by Beckman et al. [24]. Responses in the DHR assay to a standard amount of peroxynitrite were determined at different concentrations of oxygen, varying from 0 to 240 μM. Responses varied linearly over this range, corresponding to an increase in molar efficiency of rhodamine production from 32% to 45%. Rates of peroxynitrite production were corrected accordingly.

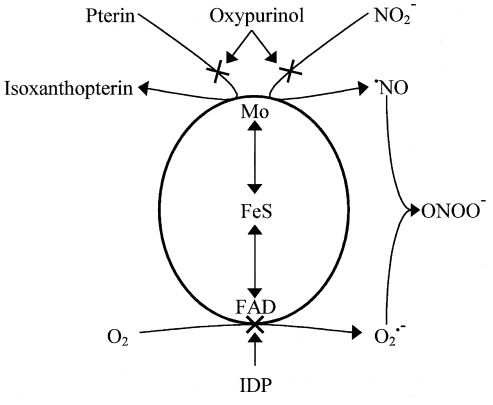

Fig. 1. Schematic representation of the redox centres of XOR. Most reducing substrates, including pterin, donate electrons to the molybdenum site (Mo), following which electrons are rapidly distributed between Mo, FAD and the two iron–sulphur centres (shown here as a single site, Fe–S) [16]. Nitrite and molecular oxygen, reduced at the Mo or FAD sites, respectively, effectively compete for a common pool of electrons. The products, NO and superoxide, then react at diffusion limited rates to give peroxynitrite [11]. Oxypurinol acts at the Mo site to inhibit both pterin oxidation and nitrite reduction. IDP acts at the FAD site inhibiting only reduction of molecular oxygen.

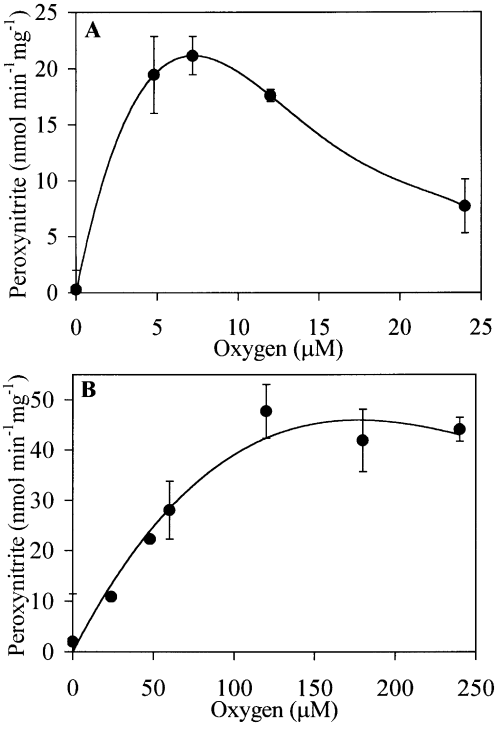

Fig. 2. Rates of peroxynitrite production versus oxygen concentration in the presence of XO (A) or XDH (B). XO (0.45 μM) or XDH (0.2 μM) were incubated, in the presence of 80 mM sodium nitrite and 100 μM DHR, in 50 mM potassium phosphate, pH 7.2, at 25.0 ± 0.2°C with varying concentrations of oxygen. Reactions were started by the addition of 40 μM pterin. Peroxynitrite production was determined by monitoring the oxidation of DHR at 500 nm, as described in Section 2. Values are means ± S.D. ($n = 3$).

2.5. Determination of oxygen concentrations

Defined concentrations of oxygen were obtained by mixing oxygen-free nitrogen with air using Sierra mass flow controllers (Sierra Instruments Inc., Monterey, CA, USA). Dissolved oxygen concentrations were checked by using a Clark type oxygen electrode, and were calculated assuming that air-saturated buffer corresponded to 240 μM.

3. Results

3.1. Peroxynitrite production catalysed by XOR in the presence of pterin and inorganic nitrite

Previous studies of XOR-catalysed reduction of inorganic nitrite [15,16] were done in the presence of xanthine or NADH as reducing substrates. However, preliminary experiments using a standard peroxynitrite generating system, SIN-1 [25], established that both urate and NADH acted as scavengers of peroxynitrite, invalidating their use in its assay. On the other hand, neither pterin nor its oxidation product, isoxanthopterin, interfered significantly with the DHR assay. Pterin was accordingly chosen as reducing substrate in the present work.

Incubation of XOR with inorganic nitrite and pterin showed rates of peroxynitrite production (Section 2) that were proportional to concentration of enzyme and could be completely inhibited by oxypurinol [26] or IDP [16,27], inhibitors of the molybdenum and FAD sites of the enzyme, respectively (Fig. 1). Neither oxypurinol nor IDP inhibited peroxynitrite generation by SIN-1 in the same system.

The dependence of peroxynitrite production catalysed by

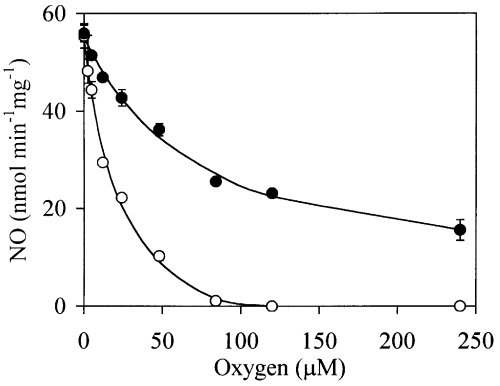

Fig. 3. Rates of NO production versus oxygen concentration in the presence of XDH, with and without addition of SOD. XDH (68.2 nM) was incubated in the presence of 40 mM sodium nitrite and 40 μM pterin in 50 mM potassium phosphate buffer, pH 7.2, at 25.0 ± 0.2°C with varying concentrations of oxygen without (○) and with (●) addition of SOD (200 U/ml). NO production was monitored in a chemiluminescence NO meter (Section 2). Values are means ± S.D. Where error bars are not shown, their width is less than that of the symbol.

XO and by XDH at varying concentrations of oxygen are shown respectively in Fig. 2A,B. The profiles for the two forms of the enzyme differ markedly in that XO-catalysed rates are slower and strongly inhibited at relatively low oxygen concentrations. XDH-catalysed rates, on the other hand, continue to increase up to approximately 200 μM oxygen.

3.2. *NO production catalysed by XOR in the presence of pterin and inorganic nitrite: effect of SOD*

The effect of oxygen concentration on NO production catalysed by XDH is shown in Fig. 3. Apparent (i.e. detectable) production of NO decreases as oxygen tension increases, falling essentially to zero at 100 μM oxygen. In the presence of 4.14 μM SOD (200 U), however, NO production is seen to be maintained at significant levels up to at least 240 μM oxygen. With XO, in contrast, NO production, although essentially the same as with XDH under anaerobic conditions, fell sharply with increasing oxygen, reaching zero at 5–6 μM oxygen in the absence of SOD. In the presence of 4.14 μM SOD, levels were higher but nevertheless reached zero at less than 40 μM oxygen (data not shown).

4. Discussion

In our previous study of XO-catalysed reduction of inorganic nitrite to NO, we briefly reported that NO production decreased with increasing concentrations of oxygen [16]. On the basis of the effects of SOD, we attributed these decreases to reaction of NO with XO-generated superoxide and presumed that the product was peroxynitrite. We now report direct demonstration of peroxynitrite production catalysed by XO and, more effectively, by XDH.

Peroxynitrite was determined by monitoring oxidation of DHR by an essentially established procedure [23,28]. In the previous work, referred to above, NADH and xanthine were used as reducing substrates [16]. These, however, proved to be impractical in the present studies because both NADH and urate acted as scavengers of peroxynitrite. The problem with urate has been noted previously [28] and surmounted by using pterin instead of xanthine as reducing substrate [9]. The present work confirmed that neither pterin nor its oxidation product, isoxanthopterin, interfered in the peroxynitrite assay. The molar efficiency of rhodamine production in the DHR assay was found to increase slightly as oxygen tensions increased, consistent with the mechanism of peroxynitrite-induced oxidation of DHR proposed by Kooy et al. [28], and was taken into account in calculation of the rate of peroxynitrite production (Section 2).

In the presence of pterin, inorganic nitrite and oxygen, XO was found to catalyse production of peroxynitrite, the rate of which peaked at approximately 7 μM oxygen and thereafter decreased with increasing oxygen concentrations (Fig. 2A). The behaviour of XDH in the corresponding system differed markedly, in that rates of peroxynitrite production continued to increase up to approximately 200 μM oxygen, achieving values some 2-fold higher than in the case of XO (Fig. 2B). These differences can be largely explained in terms of production of NO, the essential precursor of peroxynitrite. Under anaerobic conditions, rates of NO production in the presence of XO or XDH are essentially comparable. As oxygen levels increase, levels of NO fall much more rapidly in the case of XO. In the presence of SOD, rates of NO production are higher with both forms of the enzyme, consistent with removal of superoxide, which reacts with NO. This reaction ($^{\bullet}NO + O_2^{\bullet -} \rightarrow OONO^-$) generates peroxynitrite. Indeed, for XDH, the differences between the two curves, with and without SOD (Fig. 3), broadly correspond to the profile of peroxynitrite production (Fig. 2B).

Clearly, the question arises as to why NO production falls off with increasing oxygen levels so much more quickly in the case of XO. This can be at least qualitatively explained in terms of competition between nitrite and molecular oxygen for pterin-generated electrons (Fig. 1). It is well established that reducing substrates, with the exception of NADH, donate electrons to the molybdenum centre of XOR, while molecular oxygen accepts electrons at the FAD site [18]. Inorganic nitrite, on the other hand, accepts electrons directly from the molybdenum site [16]. XO [29] is known to reduce molecular oxygen some six times faster than does XDH [30], implying that XO will have less electrons available for reduction of nitrite than will XDH; a situation that will be exacerbated as oxygen tensions increase [31].

The salient observation is that in the presence of oxygen, inorganic nitrite and a reducing substrate, both XO and XDH, in effect, catalyse the production of peroxynitrite. Moreover, in the case of XDH, such production continues at significant levels even at 240 μM oxygen (i.e. aerobic conditions). Concerning the implications of these findings for the vasculature, XOR is known to occur in endothelial cells, both intra- and extracellularly [32], most probably as XDH or XO, respectively [33]. Attention has also been increasingly directed to circulating enzyme, levels of which are normally low [34] but which can rise dramatically in a number of inflammatory disease states, particularly those involving liver damage [35–37]. Peroxynitrite, from whatever source, has the capacity to effect endothelial injury [38] and may be seen as particularly relevant to cardiovascular disease. Indeed, levels of both XOR [39] and anti-XOR antibodies [40] are known to be elevated in plasma of atherosclerosis patients and the presence of the enzyme has been demonstrated in their vessel walls and plaques [41,42].

We have previously made a case for a bactericidal role in

the digestive tract for XOR, present in intestinal epithelial cells or, in the case of the neonate, derived from maternal milk [16]. This case was based on the enzyme's capacity to generate NO. It was supported by literature reports that nitrite levels in the immediate vicinity of enteric bacteria could achieve millimolar concentrations and that XOR has affinity for acidic polysaccharides such as occur in bacterial capsules. Similar and even stronger arguments apply to XOR-derived peroxynitrite, the bactericidal activities of which are better known than those of NO [9]. Moreover, as shown here, XDH, the predominant form both in intestinal epithelial cells [33] and in freshly expressed milk [43], is capable of peroxynitrite generation across a very wide range of oxygen tensions, encompassing all situations likely to be encountered physiologically. These arguments potentially provide an answer to the long-standing question as to the role of high levels of XOR in milk from all sources.

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