# Peroxynitrite formation from the simultaneous reduction of nitrite and oxygen by xanthine oxidase

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**Abstract** **One electron reductions of oxygen and nitrite by xanthine oxidase form peroxynitrite. The nitrite and oxygen reducing activities of xanthine oxidase are regulated by oxygen with $K_{oxygen}$ 26 and 100 μM and $K_{nitrite}$ 1.0 and 1.1 mM with xanthine and NADH as donor substrates. Optimal peroxynitrite formation occurs at 70 μM oxygen with purine substrates. Kinetic parameters: $V_{max}$ ~50 nmol/min/mg and $K_m$ of 22, 36 and 70 μM for hypoxanthine, pterin and nitrite respectively. Peroxynitrite generation is inhibited by allopurinol, superoxide dismutase and diphenylene iodonium. A role for this enzyme activity can be found in the antibacterial activity of milk and circulating xanthine oxidase activity.**



*Key words:* Xanthine oxidase; Peroxynitrite; Nitric oxide; Superoxide

## 1. Introduction

Peroxynitrite has been shown to react with various target molecules in biologically important systems [1,2]. Formation occurs from the diffusion limited reaction of nitric oxide (NO) and superoxide radicals [3]. Peroxynitrite causes DNA damage [4], enzyme inhibition [5], apoptosis [6] and bacterial toxicity [7,8] and has been implicated in various diseases. Therefore an understanding of the systems most likely to produce the oxidant is important to determine its role in pathology.

NO can be generated from the NO synthases (NOS), nitrite disproportionation accelerated by acid conditions [9] and bioactivation of therapeutic nitrates [10]. Superoxide is formed from the one electron reduction of oxygen by NADH/NAD(P)H oxidases [11], xanthine oxidoreductase [12] and mitochondrial electron transport enzymes [13].

Xanthine oxidase (XO) (EC 1.1.3.22) is a multi-substrate, multifunctional enzyme. High concentrations are found in mammalian milk and liver, expressed in the vascular endothelium and measured in the circulation [14–16]. Enzyme expression is regulated by inflammatory cytokines, hormones and oxygen concentration particularly in the endothelium [17]. XO is a molybdenum, flavin, iron–sulphur containing protein of the mononuclear molybdenum oxygen oxidoreductase family [18]. We were the first to show that the reduction of nitrate and nitrite by XO in the presence of NADH as reducing substrate and in the absence of oxygen leads to the production of NO [19]. However, in the presence of air saturated buffer, it was not possible to measure NO formation unless an inhibitor of superoxide generation, diphenylene iodonium (DPI), was included in the reaction [20] and it was postulated that the competition for available electrons between oxygen and nitrate/nitrite caused the loss of NO generating activity. The enzyme can utilise oxygen and also turnover using alternative acceptor substrates particularly nitrite.

This investigation has been prompted by the growing literature on circulating XO and its systemic activities. It is released into the circulation in liver failure and has been implicated in immunity to trypanosomes in certain animals [21]. Using varied oxygen concentrations, ozone enhanced NO chemiluminescence and spectrophotometry, the kinetic parameters of peroxynitrite formation has been determined. The micro-environmental conditions under which this activity occurs are important in describing the function of this enzyme in the circulation.

## 2. Materials and methods

### 2.1. Materials

Bovine XO from buttermilk (xanthine:oxygen oxidoreductase, EC 1.1.3.22, 1.4 U/mg) was from Biozyme, UK. Sodium nitrite, hypoxanthine, allopurinol, hydrogen peroxide, sodium hydroxide, β-nicotinamide adenine dinucleotide reduced form disodium salt (NADH), diethylenetriaminepentaacetic acid (DTPA), cytochrome *c* (Cyt *c*) from horse heart and dihydrorhodamine-123 (DHR) were from Sigma, UK. DPI was from ICN Biomedicals, USA. Superoxide dismutase (SOD, superoxide:superoxide oxidoreductase, EC 1.15.1.1, 5000 U/mg), from bovine erythrocytes, was from Roche, UK.

### 2.2. NO measurement

NO was measured as described by Millar et al. [20] using an ozone enhanced chemiluminescence assay in a continuous flow apparatus (Sievers NOA 280). The mean concentration of NO in the ozone chamber, derived from readings taken every second, is displayed as parts per billion (ppb), calibrated by using a known concentration of NO in nitrogen (BOC, UK). The apparatus was modified to allow a constant stream of nitrogen, mixed, as necessary, with defined concentrations of oxygen to flow over the surface of a stirred reaction mixture (1 ml). Peak NO values minus background levels were used, together with the measured gas flow rate, to calculate the molar production of NO. Oxygen concentrations were varied by blending 100% oxygen with the nitrogen carrier gas and calculating oxygen concentrations from partial pressure measurements and assuming oxygen solubility in saline as 1.5 μM/Torr [22]. This was confirmed using a calibrated Clark-type oxygen microelectrode (Strathkelvin Instruments, UK).

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### 2.3. Superoxide measurement

Superoxide generation was measured following the SOD inhibitable reduction of horse heart Cyt *c* (40 μM) at 550 nm over time in the presence or absence of 200 U/ml SOD [23]. This was performed at a range of oxygen concentrations in a 3 ml stoppered quartz cuvette adapted to allow mixed gas additions at 37°C in a spectrophotometer (Hitachi U-2010, Hitachi, UK) with stirring. Rates were converted to molarity using an extinction coefficient of 21 000 $M^{-1}$ $cm^{-1}$.

### 2.4. Urate production

Urate production, from xanthine, was followed spectrophotometrically in a stoppered cuvette at 295 nm using an extinction coefficient of $1.1\times10^4$ $M^{-1}$ $cm^{-1}$ [24].

### 2.5. NADH oxidation

NADH oxidation was also followed in the same system at 340 nm using an extinction coefficient of 6.3 $mM^{-1}$ $cm^{-1}$ [25].

### 2.6. Peroxynitrite measurement

Peroxynitrite generation was measured using the oxidation of DHR to rhodamine as described by Kooy et al. [26] and Crow [27]. The absorbance of rhodamine was measured in triplicate at 500 nm using a 96 well plate format and reader fitted with the appropriate filters, temperature controlled to 37°C (Anthos Lucy 1, Anthos instruments, Austria). Using a range of substrates and inhibitors and pregassed media, in the presence of 100 μM DTPA, kinetic parameters were calculated from progress curves using the molar extinction coefficient 78 800 $M^{-1}$ $cm^{-1}$ and correcting for the efficiency of oxidation using authentic peroxynitrite as described [26].

Values are expressed as mean ± S.E.M. of at least three repeated measurements. Kinetic fits to the experimental data were performed on a PC using GraphPad Prism version 3.02 for Windows (GraphPad Software, USA).

## 3. Results

Urate formation, NADH oxidation and superoxide generation all showed saturation kinetics dependent on the concentration of dissolved oxygen in the reaction mixture (Fig. 1A,B). With xanthine, saturation occurred within the atmospheric range compared to NADH which required oxygen supersaturation and produced apparent kinetic parameters detailed in Table 1.

In the absence of oxygen, using spectrophotometry, saturation kinetics were observed for nitrite with either NADH or xanthine as electron donors (Fig. 1C,D and Table 1) respectively. Using ozone chemiluminescence at a range of nitrite concentrations, the NO generated by XO in the presence of xanthine and the absence of oxygen was 15, 46 and 112 nmol/min/mg for 0.1, 0.5 and 1.0 mM nitrite respectively. As the oxygen concentration was increased in the reaction chamber the measurable NO signal was reduced for all concentrations of nitrite until there was no detectable NO signal (Fig. 2A).

At 70 μM oxygen, the effect of superoxide dismutase on NO generation was measured. SOD addition increased NO detection in a dose dependent manner that saturated at around 200 U/ml SOD. Generation of NO in the presence of SOD was equivalent to 31% of the value obtained in the absence of oxygen at equimolar concentrations (Fig. 2B). Further to this (Fig. 2C) DPI is shown to be an effective inhibitor of superoxide generation from xanthine and oxygen in air saturated buffer and NO generation at 70 μM oxygen was increased in a dose dependent manner (Fig. 2D). At concentrations of DPI of 400 and 600 μM the NO generated was 103% and 96% of the NO generated in the absence of oxygen (Fig. 2D).

In accordance with others [28] we noted that NADH had an inhibitory effect on spontaneous high nitrite mediated DHR oxidation in a dose dependent manner. Therefore, NADH was not used in the following experiments. Urate formation from xanthine is suggested as a scavenger of peroxynitrite and initial assays showed this to be the case, therefore hypoxanthine or pterin was used as the electron donor substrate for the following experiments.

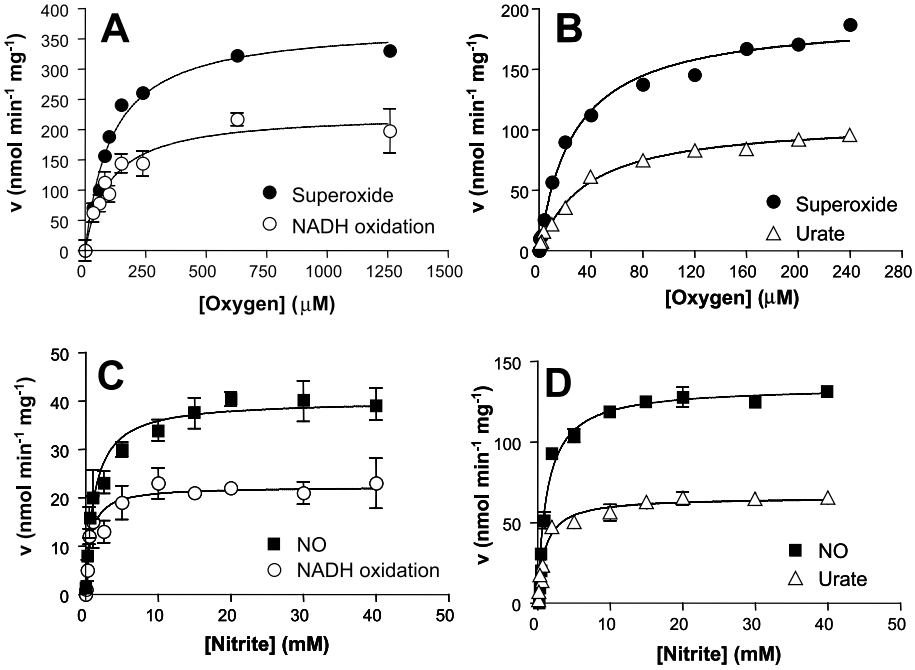


Fig. 1. The oxygen dependent superoxide, urate and NADH oxidation by XO. The rates of superoxide (●) formation and NADH (300 μM) (○) oxidation (A) and superoxide and urate (△) formation (B) from xanthine (20 μM) was determined as described. Rates of NO (■) formation were also determined in the absence of oxygen and in the presence of varying nitrite concentrations for both NADH (C) and xanthine (D) as reducing substrates. All reactions were carried out at 37°C.

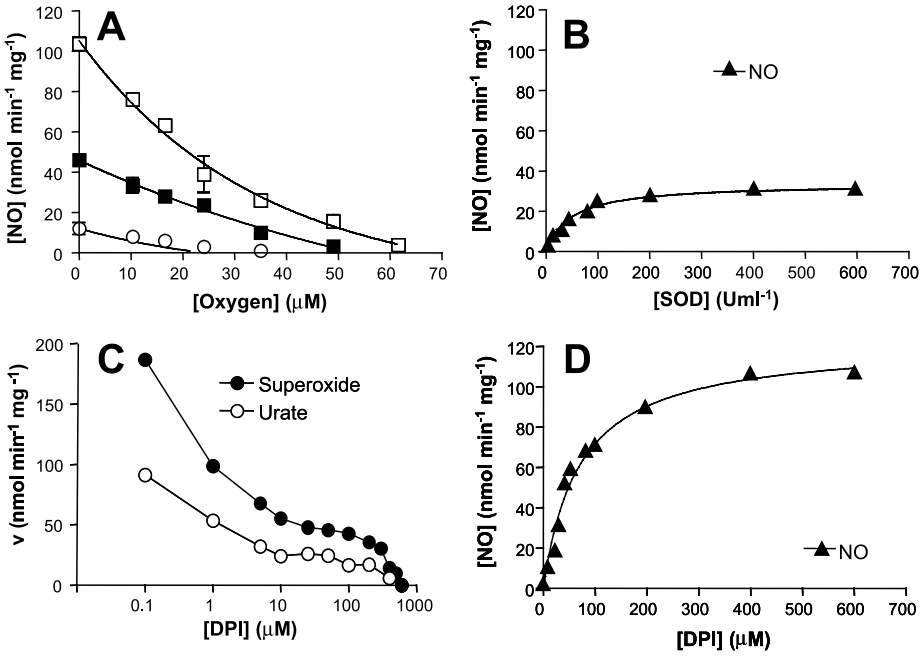


Fig. 2. The effect of oxygen, SOD and DPI on XO generated NO measured by ozone enhanced chemiluminescence. A: NO was generated from nitrite (1 □, 0.5 ■ and 0.1 mM ○) and XO from xanthine and oxygen. B: The effect of DPI on superoxide (●) and urate (○) formation from xanthine (20 μM) at 240 μM oxygen in the absence of nitrite is shown. Using 1 mM nitrite and 20 μM xanthine at 70 μM oxygen, SOD (C) or DPI (D) was added into the reaction mixture and the resultant generated NO (▲) was measured as described.

DHR oxidation was elicited from solutions containing nitrite, electron donor and enzyme (Fig. 3A) but was not seen with nitrite alone under the constraints of concentration used here or in the absence of nitrite. Rates of oxidation were taken from the progress curves and corrected for the efficiency of oxidation compared to authentic peroxynitrite. Allopurinol inhibited DHR oxidation by XO in a dose dependent manner in a system utilising 20 μM hypoxanthine and 1 mM nitrite (Fig. 3B) to give an inhibitory concentration $IC_{50}$ of ~7 μM.

The effect of oxygen concentration on the oxidation of DHR was pronounced. DHR oxidation was shown to be greatest at approximately 70–80 μM (Fig. 4A), at high and low oxygen concentrations, there was little oxidation of DHR. Kinetic parameters for nitrite, hypoxanthine or pterin at 70 μM oxygen concentration were determined and detailed in Fig. 4B,C and Table 2. Classic Michaelis–Menten kinetics are shown for both reducing substrates and nitrite in the production of peroxynitrite.

## 4. Discussion

It is clear that XO can simultaneously reduce oxygen and nitrite from various reducing and oxidising substrates with the products going on to form peroxynitrite. Under the conditions of oxygen concentration used here, rate equations and $K_m$ values can be derived for nitrite and purine substrates at 70 μM oxygen and suggest that these activities may occur most readily under physiological oxygen concentrations leading to a defined role for a circulating form of this enzyme.

Nitrite is shown to be an effective electron acceptor substrate for XO and gives comparable $K_m$ values to those described by Li et al. [29] for both NADH and xanthine as reducing substrates. However, as oxygen is allowed into the reaction, NO rates fall to zero. NO reacts rapidly with oxygen in the gaseous phase at high NO concentrations giving the characteristic brown fumes of nitrogen dioxide [30] and this may account for the loss of NO signal in these experiments. However, in the presence of SOD or DPI, superoxide concentrations are reduced allowing the return of the NO signal even in the presence of oxygen suggesting the reaction of NO and oxygen in the gaseous phase has minimal effect in this system. The reaction of NO and molecular oxygen in the aqueous phase may also have been a confounding factor but has been reported to be slow with a half-life of several hours [31]. These results suggest that simultaneous generation of NO and superoxide occurs over a range of oxygen concentrations with the maximum rates shown at around 70 μM oxygen. From the work of Beckman et al. [32] and Kissner et al. [3] the reaction of NO and superoxide is shown to be diffusion limited and the most likely reaction to occur as shown by the oxidation of DHR under the conditions described.

Physiological dissolved plasma oxygen concentrations range

Table 1
The kinetic parameter determined for XO derived urate, superoxide, NO and NADH oxidation

| | $K_{O_2}$ (μM) | $V_{app}$ (nmol/min/mg) | | | $K_{NO_2^-}$ (mM) | $V_{app}$ (nmol/min/mg) | | |
|---|---|---|---|---|---|---|---|---|
| | | Superoxide | NADH oxidation | Urate | | NO | NADH oxidation | Urate |
| Xanthine (20 μM) | 26.82 ± 1.12 | 193.2 ± 2.03 | – | 107.9 ± 0.98 | 1.039 ± 0.05 | 134.4 ± 3.32 | – | 65.84 ± 2.04 |
| NADH (300 μM) | 102.6 ± 0.56 | 374.6 ± 1.66 | 227.2 ± 3.26 | – | 1.182 ± 0.04 | 40.12 ± 2.87 | 22.31 ± 1.61 | – |

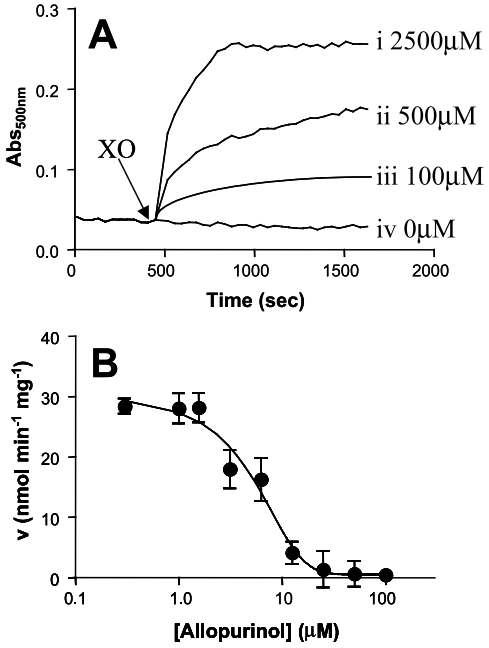


Fig. 3. XO derived DHR oxidation to rhodamine and the effect of allopurinol enzyme activity. A: DHR oxidation, in the presence of varying nitrite concentrations (i–iv) at 70 μM oxygen, with hypoxanthine (20 μM) was measured at 500 nm and 37°C. B: To this system allopurinol (●) was added at varying concentrations with 1 mM nitrite and 20 μM hypoxanthine.

from 120–135 μM on the arterial side to 55–70 μM on the venous side [33]. Plasma nitrite can be generated from nitrate reduction, the reaction of NO with oxygen and Cyt *c* catalysed formation of nitrite from NO [34,35] and ranges from 10 to 50 μM in normal adults but can rise to 130 μM in patients

Table 2
Kinetic parameters derived for the XO dependent formation of peroxynitrite

| | $K_m$ (μM) | $V_{max}$ (nmol/min/mg) Peroxynitrite |
|---|---|---|
| Hypoxanthine | 22.35 ± 0.19 | 46.11 ± 1.02 |
| Nitrite | 68.91 ± 3.66 | |
| Pterin | 36.14 ± 1.04 | 29.76 ± 0.34 |
| Nitrite | 40.13 ± 6.31 | |

Measurements were performed at 70 μM oxygen with varying nitrite and purine substrates.

with sepsis [36]. This suggests that peroxynitrite formation from XO can occur under normal physiological concentrations of substrate. Although the proportion of the XO dependent peroxynitrite formation is yet to be determined, evidence of 3-nitrotyrosine formation in healthy adults of ~130 nM suggests that normal physiological processes can lead to the formation of peroxynitrite in plasma [37].

A possible problem exists for a functional peroxynitrite forming enzyme utilising xanthine in the form of the reported scavenging activity of the product urate. Scavenging of peroxynitrite by uric acid has been suggested to decrease peroxynitrite neurotoxicity in diseases such as multiple sclerosis [38]. However, the reaction of peroxynitrite with uric acid has given conflicting results. Skinner et al. [39] showed that peroxynitrite and uric acid react to give a NO donor species having downstream effects on vascular relaxation whereas the reaction of peroxynitrite with $CO_2$ was shown to be 920 times faster than peroxynitrite with uric acid at physiological $CO_2$ concentrations [40]. The picture is further complicated with peroxynitrite permeation of erythrocytes in the presence of $CO_2$ [41]. The suggestion is therefore that the simultaneous formation of uric acid and peroxynitrite by XO does not

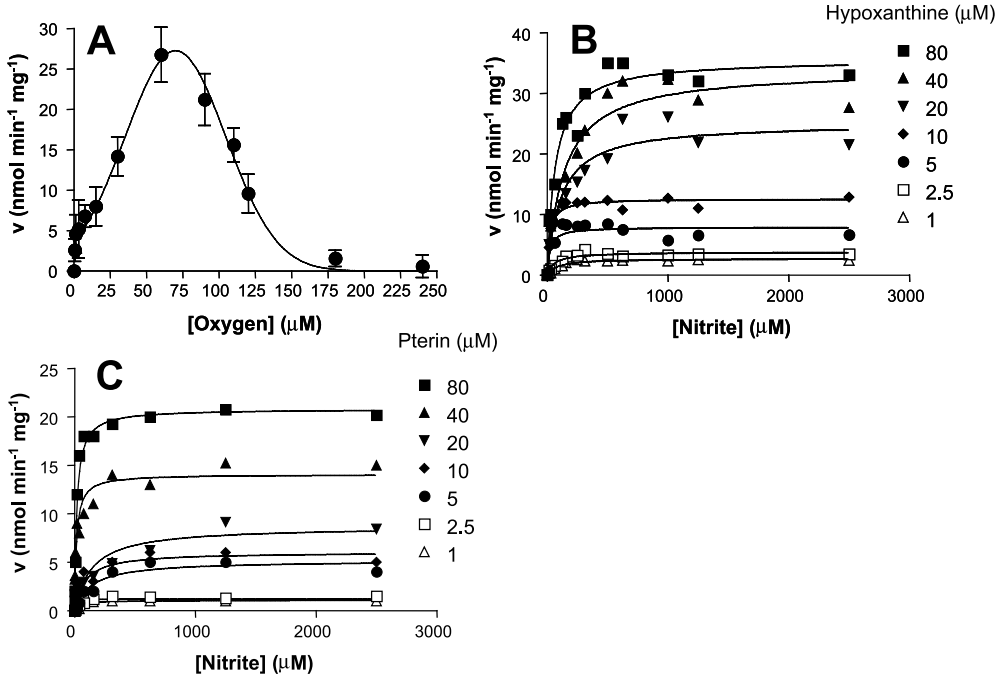


Fig. 4. The effect of oxygen and substrate concentration on XO mediated DHR oxidation. A: Using XO, 1 mM nitrite, 20 μM hypoxanthine at varying oxygen concentrations, the rate of DHR oxidation (●) was measured as described. B: Hypoxanthine at varying concentrations against varying nitrite concentrations as described at 70 μM oxygen. C: Pterin at varying concentrations against varying nitrite concentrations as described at 70 μM oxygen.

automatically lead to scavenging in physiological environments due to the competing reactions. Further to these observations, using NADH as the donor substrate can lead to the simultaneous reduction of nitrite and oxygen by XO and derive peroxynitrite in the complete absence of purines and urate.

Circulating plasma XO has been shown to be responsible for the development of endothelial dysfunction and for remote organ injury of the lung and intestine after ischaemia–reperfusion protocols [42]. We have also shown that XO can bind to bacterial surface structures and can kill bacteria by the formation of peroxynitrite both in vitro and in vivo in calves ([2,8], Millar, unpublished results). It is intriguing therefore to suggest that XO can generate peroxynitrite in the circulation, at sites systemic to its production and in the neonatal gut as a first line antibacterial defence mechanism showing a range of functions both deleterious and beneficial. The importance of the in vivo activity of this enzyme warrants further consideration in the light of these findings.

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