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Peroxynitrite Decay in the Presence of Hydrogen Peroxide, Mannitol and Ethanol: A Reappraisal

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We have reported previously that the apparent rate of peroxynitrite ($ONOO^-$) decay, as followed from its absorbance at 302 nm, decreases in the presence of hydrogen peroxide, mannitol and ethanol (Alvarez *et al.*, 1995, *Chem. Res. Toxicol.* 8:859–864; Alvarez *et al.*, 1998, *Free Radic. Biol. Med.* 24:1331–1337). Recently, two papers confirmed the observation and proposed that this slowing effect was due to the formation of absorbing peroxynitrate (O_2NOO^-) as intermediate (Goldstein and Czapski, 1998, *J. Am. Chem. Soc.* 120:3458–3463; Hodges and Ingold, 1999, *J. Am. Chem. Soc.* 121:10695–10701). Peroxynitrate would be formed from the reaction of peroxynitrite-derived nitrogen dioxide with superoxide. Superoxide, in turn, would arise from the one-electron oxidation of hydrogen peroxide, or from the reaction of reductive radicals derived from mannitol and ethanol with dioxygen. In agreement with this concept, we show herein that under the conditions of our previous work, the slowing effect is prevented by superoxide dismutase and, in the case of mannitol and ethanol, by reducing the dioxygen concentration of the reaction solutions. Thus, superoxide formation is necessary for the decrease in the rate of absorbance decay. In addition, by simulations using known rate constants and absorption coefficients, we show that the slowing effect can be quantitatively accounted for by the formation of peroxynitrate.

Keywords: peroxynitrite, superoxide, peroxynitrate, hydrogen peroxide, mannitol, ethanol, oxoperoxonitrate(1-), dioxoperoxonitrate(1-)

INTRODUCTION

Peroxynitrite† is the product of the reaction between nitric oxide ($\cdot NO$) and superoxide ($O_2^{\cdot -}$) radicals. The anion is relatively stable. When protonated to peroxynitrous acid ($pK_a = 6.8$), it decays in a first order process yielding nitrate ($k = 1.2\ s^{-1}$ at 25°C)[1]. The pH-dependent decay of peroxynitrite occurs through homolysis of the peroxidic oxygens to hydroxyl ($\cdot OH$) and nitrogen dioxide radicals ($\cdot NO_2$). The radicals are initially formed in a cage of solvent molecules, and can either recombine inside the cage or diffuse

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† The term peroxynitrite is used to refer to both peroxynitrite anion and peroxynitrous acid. IUPAC recommended names are oxoperoxonitrate(1-) for peroxynitrite, hydrogen oxoperoxonitrate for peroxynitrous acid, dioxoperoxonitrate(1-) for peroxynitrate and hydrogen dioxoperoxonitrate for peroxynitric acid.

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out of it. The free radicals formed in approximately 30 % yield can oxidise different target molecules[2–9].

In previous papers we characterised the reactions of peroxynitrite with hydrogen peroxide, mannitol and ethanol[10, 11]. In these reactions, the oxidising species was hydroxyl radical derived from peroxynitrite. In the case of hydrogen peroxide, hydroxyl radical would oxidise hydrogen peroxide to dioxygen, with the intermediate formation of superoxide. The amount of peroxynitrite that yielded hydroxyl radical was estimated as 32 %, and the amount of dioxygen formed in the reaction was inhibited by several hydroxyl radical scavengers to the extent predicted by their rate constants with hydroxyl radical. As for the reaction of peroxynitrite with mannitol and ethanol, dioxygen consumption was observed and, in the case of ethanol, formation of aldehydes and α- and β-hydroxyethyl radicals[3,5].

The kinetic studies showed that peroxynitrite decay, followed through the decrease in absorbance at 302 nm, slowed in the presence of hydrogen peroxide, mannitol or ethanol. These results were tentatively interpreted as indicative of the formation of stabilising complexes between the substrates and peroxynitrite. Recently, through experiments mainly performed in the presence of high concentrations of carbon dioxide, where peroxynitrite yields $CO_3^{\cdot -}$ and $\cdot NO_2$[12–14], two groups have independently confirmed this slowing effect, and provide a different interpretation to the observed results[15, 16]. According to these authors, the apparent slowing would be due to the formation of peroxynitrate (O_2NOO^-) as intermediate: $\cdot NO_2 + O_2^{\cdot -} \rightarrow O_2NOO^- \rightarrow O_2 + NO_2^-$

The nitrogen dioxide formed from the homolysis of peroxynitrite reacts with superoxide yielding peroxynitrate. Superoxide would arise, in the case of hydrogen peroxide, from the one-electron oxidation of hydrogen peroxide, and in the case of mannitol and ethanol, from the reaction with dioxygen of reductive radicals derived from their one-electron oxidation. Peroxynitrate, in turn, decays to nitrite and dioxygen. But since peroxynitrate itself absorbs at 302 nm, it would decrease the rate of decay of the absorbance, giving rise to the apparent slowing of peroxynitrite decay. Unlike peroxynitrite, peroxynitrate is stable at acidic pH, but decays when unprotonated (pKa = 5.8–5.9) with a rate constant of 1 s^{-1}. Peroxynitrate anion absorbs in the UV, with a maximum at 285 nm ($\varepsilon = 1650$ M^{-1} cm^{-1}), while peroxynitric acid does not absorb significantly beyond 280 nm[17,18].

In this paper, we investigated whether the slowing effect is dependent on superoxide formation under the conditions of our previous work. Thus, we measured the rate of decrease of the absorbance at 302 nm with mannitol, ethanol and hydrogen peroxide, in the presence of Cu,Zn superoxide dismutase (SOD) or reduced dioxygen concentrations to inhibit peroxynitrate formation. In addition, we performed simulations with the known reactions and absorption coefficients. Both approaches show that the apparent slowing effect is mediated by superoxide, and can be quantitatively accounted for by the formation of peroxynitrate.

MATERIALS AND METHODS

Materials

Peroxynitrite was synthesised from hydrogen peroxide and sodium nitrite as reported[2, 19]. Hydrogen peroxide was obtained from Fluka (Germany), absolute ethanol was from Biopack (Argentina), bovine Cu,Zn-SOD was from DDI Pharmaceuticals (USA), bovine milk xanthine oxidase was from Calbiochem (USA), argon was from Aga (Uruguay). All the other reagents were from Sigma (USA).

Stopped-Flow Experiments

Kinetic experiments were carried out in an Applied Photophysics stopped-flow (SF17MV), at (25.0 ± 0.1)°C according to the instrument's

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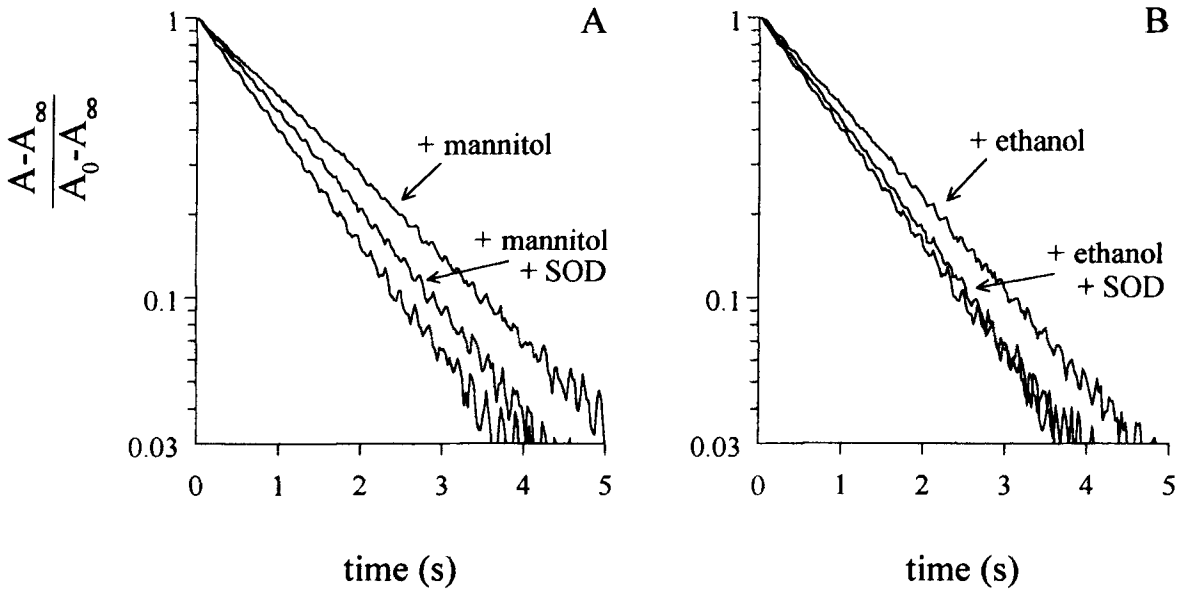

FIGURE 1 Kinetic traces of absorbance at 302 nm from peroxynitrite decay in air equilibrated solutions, in the presence of mannitol and ethanol. Peroxynitrite (0.50 mM) was mixed with phosphate buffer, 0.1 M, pH 6.30 ± 0.01, dtpa 0.1 mM, in air equilibrated solutions, in the presence or absence of 0.1 M mannitol (Fig. 1A), ethanol (Fig. 1B), and 208 U mL^{-1} of SOD

sensor. The absorbance at 302 nm was followed for 10 s and fitted to a single exponential function with the software provided with the instrument. The exponential rate constants reported are the means ± standard deviation (n≥10) of a representative experiment, and the experiments were performed at least three times with results that differed in less than 8 %. To reduce the dioxygen concentration in some experiments, the solutions were extensively purged with argon for 15 minutes immediately before mixing in the stopped-flow. To prevent volatilisation of ethanol from the solutions, absolute ethanol was degassed separately and added to purged buffer with a gas-tight syringe.

Analytical Procedures

Contaminating nitrite was determined with the Griess reaction using sodium nitrite as standard[20,21], after decay of peroxynitrite in phosphate buffer at pH 6.3. At this pH, more than 90 % of the peroxynitrite yields nitrate[22]. Superoxide dismutase activity was measured through the method of inhibition of cytochrome *c* reduction. One SOD unit was defined as the amount of enzyme that inhibited cytochrome *c* reduction by superoxide in 50 %, where superoxide was generated by the xanthine/xanthine oxidase system[23,24].

Simulations

The reactions were simulated according to published rate constants and absorption coefficients with the software Gepasi[25,26]. The simulated time-dependent data of absorbance at 302 nm were fitted to a single exponential function with Slide Write (Advanced Graphics Software, Inc.)

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RESULTS AND DISCUSSION

Mannitol and Ethanol System

Peroxynitrite decay in phosphate buffer followed a first-order function with a rate constant of (0.931 ± 0.008) s^{-1} at pH 6.30 ± 0.01 and 25°C (Fig. 1). In the conditions of our previous work, when 100 mM mannitol or ethanol were added the apparent rate of peroxynitrite decay, as seen by its absorbance at 302 nm, decreased by 31 or 23 %, respectively. The absorbance at 302 nm decreased following an exponential function with rate constants of (0.643 ± 0.005) s^{-1} for mannitol (Fig. 1A) and (0.714 ± 0.007) s^{-1} for ethanol (Fig. 1B).

If the apparent slowing was due to the formation of peroxynitrate, it would be prevented in the presence of SOD. This enzyme would divert more superoxide towards dismutation instead of reaction with nitrogen dioxide, so that the slowing effect would tend to disappear. Indeed, when 208 U mL^{-1} of SOD were added (≈ 1.3 μM), the slowing effect was significantly suppressed both for mannitol and for ethanol (Fig. 1), and the rate of decay of the absorbance at 302 nm increased to values of (0.79 ± 0.01) s^{-1} and (0.879 ± 0.006) s^{-1}, respectively, supporting that superoxide was essential for the apparent slowing of peroxynitrite decay.

Doubling the SOD concentration increased further the rate of absorbance decay in the presence of mannitol and ethanol by ≈ 3.2 %. To check that the enzyme remained active under these experimental conditions, measurements were performed and indicated no inactivation of SOD (data not shown). Control experiments showed that SOD alone increased the rate of decay only by ≈1.6 %, indicating that suppression of the slowing effect could not be accounted for by direct reaction between SOD and peroxynitrite.

Since superoxide is needed for the slowing effect, avoiding its formation would restore the apparent rate of peroxynitrite decay to the values measured in the absence of mannitol and ethanol as well. Superoxide is formed from the reaction of reductive radicals derived from mannitol and ethanol with dioxygen. Thus, this hypothesis was tested by purging the solutions with argon before triggering the stopped-flow.

When the solutions were purged, the rate of peroxynitrite decay in phosphate buffer alone decreased by about 10 %, from (0.931 ± 0.008) s^{-1} to (0.839 ± 0.006) s^{-1}, probably because of displacement of contaminating carbon dioxide from the air-equilibrated solutions. Decreases of similar magnitude upon purging have been previously reported[27]. We can calculate from the second order rate constant between carbon dioxide and peroxynitrite anion (7200 $M^{-1}s^{-1}$ at pH 6.3 and 25°C)[12,28] that the carbon dioxide contamination was 13 μM, in accordance with previous estimations[27].

With argon purged solutions, the slowing effect was suppressed for mannitol and ethanol (Fig. 2), and the apparent rate of absorbance decay increased to (0.78 ± 0.01) and (1.01 ± 0.03) s^{-1} respectively, confirming that formation of superoxide was necessary for the apparent slowing through the formation of peroxynitrate. Addition of 208 U mL^{-1} of SOD further increased the apparent rate constant, suggesting that anaerobiosis was not complete in this system (data not shown). The fact that the rate constant with ethanol actually increased from (0.839 ± 0.006) to about (1.01 ± 0.03) s^{-1} could be revealing direct reactions of peroxynitrite with ethanol or, more likely, the α-hydroxyethyl radical.

Hydrogen Peroxide System

In the presence of 60 mM hydrogen peroxide, the apparent rate of peroxynitrite decay in air-equilibrated solutions decreased from (0.931 ± 0.008) s^{-1} to (0.764 ± 0.004) s^{-1} (Fig. 3A). SOD (208 U mL^{-1}, ≈ 1.3 μM) partially eliminated the slowing effect of hydrogen peroxide, raising the apparent rate constant to (0.819 ± 0.009) s^{-1}, and suggesting that superoxide is implicated (Fig. 3A). Again, doubling the amount of SOD further increased the rate constant by ≈ 2.6 % and con-

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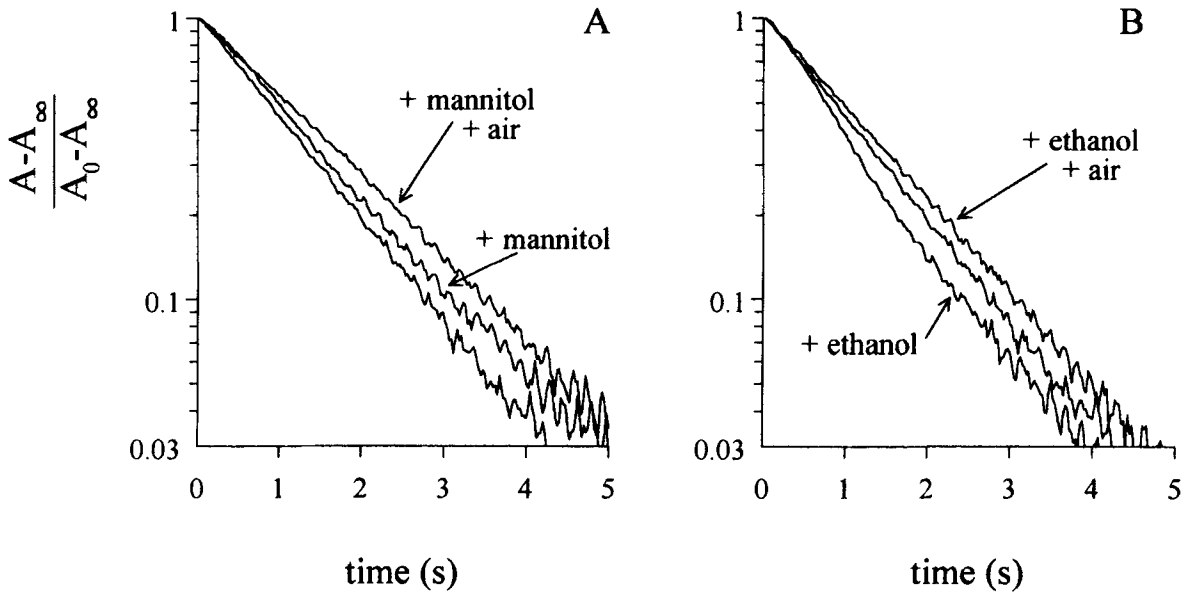

FIGURE 2 Kinetic traces of absorbance at 302 nm from peroxynitrite decay in argon purged solutions, in the presence of mannitol and ethanol. Peroxynitrite (0.50 mM) was mixed with phosphate buffer, 0.1 M, pH 6.30 ± 0.01, dtpa 0.1 mM, in argon purged solutions, in the presence or absence of 0.1 M mannitol (Fig. 2A) and ethanol (Fig. 2B). Air equilibrated solutions are included for comparison

trols showed that in these experimental conditions SOD was not inactivated (data not shown).

Remarkably, when the solutions were purged with argon, the rate of decay of the absorbance at 302 nm in the presence of hydrogen peroxide remained (0.738 ± 0.003) s^{-1}(Fig 3B). This observation is consistent with the fact that, for the hydrogen peroxide system, dioxygen is not necessary for superoxide formation. Rather, it is formed from the one-electron oxidation of hydrogen peroxide.

Simulations

To rationalise the decrease in the rate of decay of the absorbance at 302 nm in the presence of hydrogen peroxide, mannitol and ethanol, simulations were performed according to the reaction schemes shown in Table I. Time-dependent data of absorbance at 302 nm were simulated and fitted to a single exponential function. For the simulations, the known absorption coefficients of peroxynitrite anion (1670 $M^{-1}cm^{-1}$[29]) and peroxynitrate anion (estimated as 1400 $M^{-1}cm^{-1}$[17]) were used, and it was assumed that the protonated forms did not absorb. The pH was considered fixed at 6.3 and the concentration of HPO_4^{2-} was 0.024 M. The concentration of dioxygen was assumed 240 μM[30]. The initial concentration of peroxynitrite was 0.50 mM, similar to the experimental one. Nitrite was measured in different peroxynitrite stocks and found to be variable depending on the age of the stock solution, in average 1 nitrite per peroxynitrite. A limited number of experiments were reproduced under low nitrite contamination (0.3 nitrite per peroxynitrite) obtained from freshly prepared peroxynitrite solutions. For the simulations, a nitrite concentration of 0.50 mM was considered (one nitrite per peroxynitrite). It was assumed that 30 % of peroxynitrite was able to yield free hydroxyl and nitrogen dioxide radicals, with 70 % recombining inside the cage. The recombination of these free radicals[31], the reaction of hydroxyl radical with peroxynitrite anion ($k = 4.8 \times 10^9$ $M^{-1}s^{-1}$)[32] and the homolysis of

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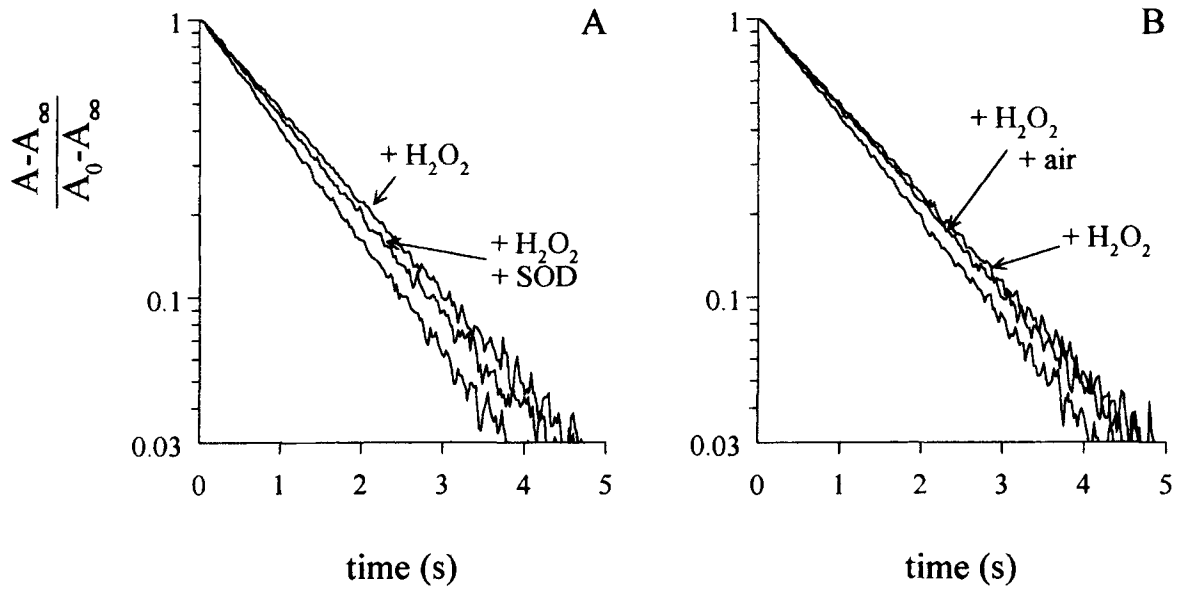

FIGURE 3 Kinetic traces of absorbance at 302 nm from peroxynitrite decay in the presence of hydrogen peroxide. Peroxynitrite (0.50 mM) was mixed with phosphate buffer, 0.1 M, pH 6.30 ± 0.01, dtpa 0.1 mM, in air equilibrated (Fig. 3A) or argon purged (Fig. 3B) solutions, in the presence or absence of 0.06 M hydrogen peroxide or 208 U mL^{-1} of SOD. In Fig. 3B air equilibrated solutions are included for comparison

peroxynitrite anion to superoxide and nitric oxide ($k = 0.017\ s^{-1}$)[33] were negligible. Dtpa was not included in the simulation, since although hydroxyl radical can react with it[15,34], at the concentration of 0.1 mM used the amount of initial peroxynitrite trapped by it was less than 2.6 % in the absence of targets, and negligible in their presence. In addition, similar results were obtained in control experiments with 10 times less dtpa. Simulations were performed for ethanol and hydrogen peroxide since for both systems the participating rate constants are well known.

The results obtained are shown in Table II. For the hydrogen peroxide system, the simulated results, obtained through equations 1–4 and 9–15, were in excellent accordance with the experimental ones, on agreement with the slowing effect being due to the formation of peroxynitrate. The simulation also allowed us to estimate the total formation of peroxynitrate in this system, which was 7.2 % of the initial peroxynitrite, on agreement with hydrogen peroxide at this concentration being able to trap only a fraction of the hydroxyl radicals formed. Simulated results fitted the experimental ones also in the presence of SOD.

For the ethanol system, the simulation according to equations 1–8 and 10–15 slightly deviated from exponentiallity and led to lower rate constants than found for hydrogen peroxide, on agreement with ethanol being able to trap most of the hydroxyl radical formed. However, the apparent rate constant found experimentally was higher than the simulated one. The fact that the observed slowing effect was not as important as expected remains to be established. Controls showed that the discrepancy was not due to nitrite, since experiments with lower concentrations of it led to similar results. It is possible that the discrepancy may be due to the fact that secondary reactions of the radicals could be occurring, such as the α-hydroxyethyl radical reacting with itself, with nitrogen dioxide or with peroxynitrite. In addition, it is possible that the intermediates could be reacting with peroxynitrate as well.

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TABLE I Reactions involved in peroxynitrite decay at acidic pH, and in the presence of hydrogen peroxide, ethanol and superoxide dismutase

Peroxynitrite decay:	
$ONOOH \rightleftharpoons ONOO^- + H^+$	pKa = 6.8[19] (1)
$ONOOH \rightarrow 0.7\ HNO3 + 0.3\cdot NO_2 + 0.3\cdot OH$	k =1.2 s^{-1}[35] (2)
$\cdot OH + NO_2^- \rightarrow OH^- + \cdot NO_2$	k = 1×10^{10} $M^{-1}s^{-1}$[34] (3)
$2\cdot NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2\ H^+$	k = 4.7×10^7 $M^{-1}s^{-1}$[34] (4)
Additional reactions in the presence of ethanol[a] :	
$\cdot OH + CH_3CH_2OH \rightarrow 0.84\ CH_3\cdot CHOH + 0.03\ CH_3CH_2O\cdot + 0.13\cdot CH_2CH_2OH + H_2O$	k = 1.9×10^9 $M^{-1}s^{-1}$[34,36] (5)
$CH_3\cdot CHOH + O_2 \rightarrow CH_3CH(OH)OO\cdot$	k = 4.6×10^9 $M^{-1}s^{-1}$[34] (6)
$CH_3CH(OH)OO\cdot \rightarrow CH_3CHO + H^+ + O_2^{\cdot -}$	k = 61 s^{-1}[34] (7)
$CH_3CH(OH)OO\cdot + HPO_4^{2-} \rightarrow CH_3CHO + H_2PO_4^- + O_2^{\cdot -}$	k = 4.0×10^6 $M^{-1}s^{-1}$[34] (8)
Additional reaction in the presence of hydrogen peroxide:	
$H_2O_2 + \cdot OH \rightarrow HO_2\cdot + H_2O$	k = 2.7×10^7 $M^{-1}s^{-1}$[34] (9)
Formation and decay of peroxynitrate:	
$\cdot NO_2 + O_2^{\cdot -} \rightarrow O_2NOO^-$	k = 4.5×10^9 $M^{-1}s^{-1}$[17] (10)
$O_2NOOH \rightleftharpoons O_2NOO^- + H^+$	pKa = 5.9[18] (11)
$O_2NOO^- \rightarrow O_2 + NO_2^-$	k = 1 s^{-1}[17] (12)
Spontaneous and SOD-catalysed dismutation of superoxide:	
$HO_2\cdot \rightleftharpoons O_2^{\cdot -} + H^+$	pKa = 4.8 (13)
$HO_2\cdot + O_2^{\cdot -} + H^+ \rightarrow H_2O_2 + O_2$	k = 1×10^8 $M^{-1}s^{-1}$[34] (14)
$O_2^{\cdot -}$ + Cu,Zn SOD $\rightarrow$ products	k = 2 3 $\times 10^9$ $M^{-1}s^{-1}$[37] (15)

a. Analogous reactions are expected to occur with mannitol.

TABLE II Experimental *versus* simulated rates of decrease in the absorbance at 302 nm

Condition	*Experimental* (s^{-1})	*Simulated* (s^{-1})
Control	0.931 ± 0.008	0.912
+ mannitol	0.643 ± 0.005	ND
+ mannitol, + SOD	0.79 ± 0.01	ND
+ ethanol	0.714 ± 0.007	0.512
+ ethanol, + SOD	0.879 ± 0.006	0.669
+ H_2O_2	0.764 ± 0.004	0.741
+ H_2O_2, + SOD	0.819 ± 0.009	0.804

The experimental values were determined by mixing peroxynitrite (0.50 mM) with phosphate buffer (0.1 M, pH = 6.30 ± 0.01, dtpa 0.1 mM) in the presence or absence of mannitol (0.1 M), ethanol (0.1 M) or hydrogen peroxide (0.06 M). The simulated values were obtained through the reactions shown in Table I. Simulated data of absorbance at 302 nm versus time were fitted to a single exponential function. ND: not determined, the effect of mannitol was not simulated because some of the rate constants involved are not known.

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The simulation allowed us to rationalise the fact that SOD was not able to inhibit the slowing effect completely for both systems. The enzyme, at these concentrations, could not outcompete nitrogen dioxide for superoxide. For example, in the presence of hydrogen peroxide, SOD trapped 42 % of the superoxide formed and nitrogen dioxide trapped 58 %. Although it was not convenient to raise the concentration of SOD too much in the experiments because proteins react with peroxynitrite, doubling the amount of SOD raised the apparent rate constant by 2.6 %, similar to the simulated value of 3.5 %.

CONCLUSIONS

The decrease in the rate of decay of the absorbance at 302 nm in the presence of hydrogen peroxide, mannitol and ethanol was found to be dependent on superoxide formation, supporting that this species is necessary for the slowing effect through the formation of peroxynitrate[15, 16]. Simulations with known reactions and absorption coefficients show that the slowing effect can be quantitatively accounted for by the formation of peroxynitrate.

Acknowledgements

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