Formation of peroxynitrite from *S*-nitrosothiols and hydrogen peroxide

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Received (in Cambridge) 25th March 1999, Accepted 16th April 1999

_S_-Nitrosocysteine reacts with hydrogen peroxide in aqueous buffer in the presence of EDTA to form the peroxynitrite anion. Spectral measurements show that at high pH (13.1) the peroxynitrite formed is relatively stable whereas at low pH (7.4) its decomposition rate is such that no peroxynitrite is observable. At an intermediate pH (11.6) it is possible to see both its formation and decay. Measured rate constants increase with the pH of the medium suggesting that reaction occurs by nucleophilic attack by the hydroperoxide ion at the nitroso nitrogen atom. By judicious choice of wavelength for rate measurements it is possible to obtain (in the pH range 10–13), both the second order rate constant for peroxynitrite formation and the first order rate constant for peroxynitrite decomposition. The latter is in reasonable agreement with the literature value. Other _S_-nitrosothiols behave similarly.

It is now believed that *S*-nitrosothiols may play a major role *in vivo* in the 'nitric oxide story', particularly with reference to the storage and transport of NO within the body.[1] We have shown that *S*-nitrosothiols generally, release NO in aqueous buffer at pH 7.4 by a pathway brought about by reaction with Cu(I), which is generated by reduction of Cu(II) by thiolate ion, both of which can be present only at low level impurity concentrations.[2] Cu(I) can also be made available when the Cu(II) is present in the form bound to peptides and proteins.[3] *S*-Nitrosothiols will also (when the copper reaction is suppressed) undergo nucleophilic substitution at the nitroso nitrogen atom, *i.e.* RSNO can act as a direct electrophilic nitrosating species, in the same way as do alkyl nitrites and *N*-nitroso compounds. Reaction with thiolate results in transnitrosation, (giving another *S*-nitrosothiol),[4] with ascorbate, NO is generated[5] and with secondary amines nitrosamines are formed.[6]

We now report that *S*-nitrosocysteine (SNCys) reacts readily with hydrogen peroxide in the presence of EDTA (which eliminates the copper ion promoted reactions) to yield the peroxynitrite anion. We observe the absorbance band centred around 302 nm which is characteristic[7] of the peroxynitrite anion with an extinction coefficient of 1670 ± 50 dm^3 mol^{-1} cm^{-1}.

It is known that peroxynitrite decomposes rapidly, particularly in acid solution to give nitrate ion, *via* peroxynitrous acid (pK_a 6.5). A detailed kinetic study of the isomerisation has been presented.[8] In alkaline solution there is another decomposition pathway[9] to give nitrite anion, probably catalysed by Cu^{2+}.

For the reaction of SNCys (generated in solution by nitrosation of cysteine, and used *in situ*) at pH 7.4, we see no evidence of peroxynitrite formation (see Fig. 1), but merely observe the hydrogen peroxide-promoted decomposition of SNCys as monitored by the decreasing absorbance at 340 nm. Under these conditions the reaction is relatively slow, but much faster than the spontaneous thermal decomposition of SNCys. In contrast at high pH (13.1), where peroxynitrite is known to be relatively stable, we see clearly the formation of the characteristic absorbance at 302 nm which occurs concurrently with the decreasing absorbance at 340 nm (see Fig. 2). The final absorbance at 302 nm at this pH value is relatively constant (on the time scale of the experiment), showing that here, as expected, peroxynitrite is fairly stable. Over a longer period however (several hours), we do get some peroxynitrite decomposition. At an intermediate pH value (11.6) we see the initial formation of peroxynitrite followed by its decomposition, again taking place concurrently with the decreasing absorbance of SNCys at 340 nm.

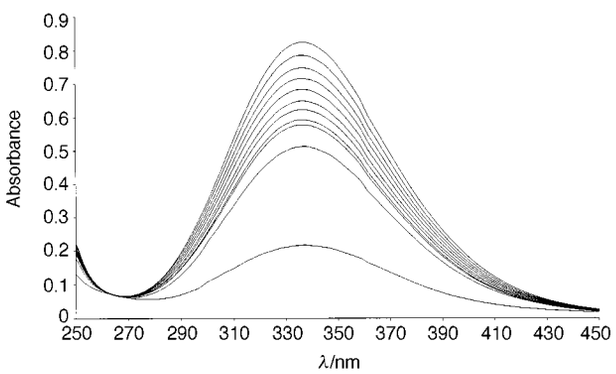

Fig. 1 Repeat scans every 20 min showing the decreasing absorbance at 340 nm due to SNCys (1×10^{-3} mol dm^{-3}) for reaction with H_2O_2 (1×10^{-2} mol dm^{-3}) at pH 7.4. The last two scans are after 4 and 24 hours respectively.

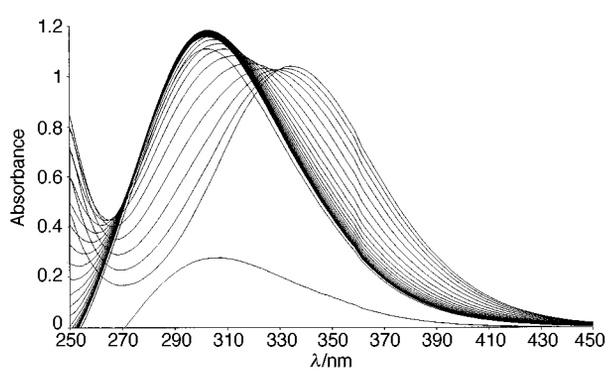

Fig. 2 Repeat scans every minute showing the decreasing absorbance at 340 nm due to SNCys (1×10^{-3} mol dm^{-3}) and the increasing absorbance at 302 nm due to $ONOO^-$ for reaction with H_2O_2 (1×10^{-2} mol dm^{-3}) at pH 13.1. The last two scans are after 100 min and 18 hours.

All rate measurements were carried out with $[H_2O_2]_o \gg [RSNO]_o$. Good first order behaviour (better than ±3%) occurred, and there was also a first order dependence on $[H_2O_2]$. The rate of disappearance of SNCys is markedly pH dependent (see Table 1), increasing with pH: this is consistent with a reaction *via* the hydroperoxide anion (pK_a 11.5) as outlined in eqn. (1), where rate-limiting nucleophilic attack by HOO^- at the nitroso nitrogen atom generates the peroxynitrite anion, which decomposes *via* its protonated form (eqns. (2) and (3)), or by another process at high pH, leading to nitrite ion formation (eqn. (4)).

Table 1 First order rate constants for the reaction of SNCys (5×10^{-3} mol dm^{-3}) with H_2O_2 (5×10^{-2} mol dm^{-3}) as a function of pH, measured at 545 nm

pH	k/s^{-1}
7.40	3.85×10^{-5} [a]
9.75	7.96×10^{-4}
11.7	3.62×10^{-3}
13.1	4.83×10^{-3}

[a] Measured at 340 nm (SNCys 1×10^{-3} mol dm^{-3}, H_2O_2 1×10^{-2} mol dm^{-3}).

Table 2 First order rate constants for the decomposition of peroxynitrite as a function of pH

pH	k/s^{-1} (measured)	k/s^{-1} (calculated) [a]
10.4	2.72×10^{-4}	$4.0 \pm 1.6 \times 10^{-4}$
11.1	5.88×10^{-5}	$7.9 \pm 2.9 \times 10^{-5}$
12.1	3.08×10^{-5}	$7.9 \pm 2.9 \times 10^{-6}$
12.8	2.20×10^{-5}	$1.6 \pm 0.6 \times 10^{-6}$

[a] From $k = 1.0\,[H^+]/([H^+] + 1.0 \times 10^{-7})$, from ref. 8.

$$RSNO + HOO^- \longrightarrow RS^- + ONOO^- + H^+ \quad (1)$$

$$ONOO^- + H^+ \rightleftharpoons ONOOH \quad (2)$$

$$ONOOH \longrightarrow NO_3^- + H^+ \quad (3)$$

$$ONOO^- \longrightarrow NO_2^- + 1/2\ O_2 \quad (4)$$

Other *S*-nitrosothiols including *S*-nitrosoglutathione and *S*-nitrosopenicillamine behaved similarly, so the reaction is quite general.

Even at high pH the formation of peroxynitrite is not quite quantitative—typical yields are ~80%. This is probably accounted for by the concurrent OH^- catalysed hydrolysis of SNCys, which is known[6] to occur at a significant rate at this pH. Also it is known[9] that peroxynitrite oxidises thiols to disulfides at pH 7.4 in a reaction which competes with the spontaneous decomposition of peroxynitrous acid. This reaction occurs *via* the free thiol form and not *via* the thiolate anion, and so this would be a relatively minor pathway for loss of peroxynitrite at high pH, but may contribute to the somewhat reduced peroxynitrite yields observed.

At high pH we can measure the decomposition rate of peroxynitrite, starting the measurements at 302 nm once all the RSNO has disappeared. We find good first order behaviour: some data are given in Table 2, together with the calculated values obtained from ref. 8. The agreement is acceptable at the lower pH values, but is much less good at pH 11.5 and above, no doubt because of the alternative decomposition pathway at high pH leading to nitrite ion formation, and which is not taken into account in the calculated *k* values in Table 2.

Peroxynitrous acid is believed to be the initial product when hydrogen peroxide reacts with nitrous acid in a mildly acid medium,[10] but rapidly isomerises to nitrate ion and a proton unless the pH is raised very rapidly. 2–Ethoxyethyl nitrite and *N*-methyl-*N*-nitrosotoluene-*p*-sulfonamide however react smoothly with hydrogen peroxide in alkaline solution to give peroxynitrite, which is relatively stable at high pH.[11] As far as we are aware, this is the first time that a *S*-nitrosothiol has been shown to yield peroxynitrite with hydrogen peroxide.

In recent years there has been much interest in peroxynitrite chemistry with regard to its possible involvement *in vivo* in the chemistry of nitric oxide (which is responsible for the control of a number of physiological functions) generated from L-arginine. It is widely believed that it is formed *in vivo* by reaction of NO with superoxide, and that (being a very powerful oxidising species) it plays a major role in cytotoxicity.[12] All the evidence is however somewhat circumstantial, and the interpretation of peroxynitrite formation and involvement *in vivo* has been challenged.[13] Nevertheless there continues to be much interest in this area, and the demonstration of peroxynitrite formation from *S*-nitrosothiols and hydrogen peroxide, both naturally occurring species, is a novel addition to the discussion.

Acknowledgements

We thank the EPSRC for a research studentship to P. J. C.

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Communication 9/02420D