Reaction of *S*-nitrosothiols with ascorbate: clear evidence of two reactions

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Ascorbate reacts with *S*-nitrosothiols at pH 7.4 *via* two pathways, in which ascorbate (at low concentration) acts as a reducing agent generating Cu^{I}, and (at high concentration) acts as a nucleophile, attacking the nitroso group.

We have shown[1] that *S*-nitrosothiols (RSNO) decompose in aqueous buffer at pH 7.4 to give nitric oxide and the corresponding disulfides, in a reaction brought about by Cu^{I} [eqn. (2)], which is generated from Cu^{II} by reduction with thiolate anion [eqn. (1)]. Often there is enough Cu^{II} present in the water/buffer system to effect reaction, and usually there is enough thiolate present in equilibrium[2] with RSNO [eqn. (3)] to bring about the reduction. In addition it has been shown[3] that Cu^{II} bound in proteins, peptides and amino acids can also be reduced by thiolate and effect RSNO decomposition, so that it is not necessary for the Cu^{II} to be present as free solvated Cu^{2+}. It follows that other reducing agents should be capable of the copper reduction, and it has been shown[1] that added ascorbate does indeed behave in a similar fashion to added *N*-acetylpenicillamine in the decomposition of *S*-nitroso-*N*-acetylpenicillamine, but hitherto no detailed mechanistic study has been carried out.

$$2Cu^{2+} + 2RS^{-} = 2Cu^{+} + RSSR \quad (1)$$

$$Cu^{+} + RSNO = Cu^{2+} + RS^{-} + NO \quad (2)$$

$$RSH + HNO_2 = RSNO + H_2O \quad (3)$$

Recently, however, it has been reported, (i) that ascorbate will promote NO release from *S*-nitroso albumin and *S*-nitroso glutathione (GSNO) in blood plasma even in the presence of metal chelating agents,[4] and (ii) in a separate study[5] using plasma and fractions of liver and kidney extracts, it is also claimed that ascorbate promotes the decomposition of GSNO, again in the absence of copper ions. A further paper in the biological literature[6] found glutathione and nitrite as the products of the reaction of GSNO with ascorbate. These findings prompted us to examine further the effect of ascorbate on RSNO decomposition.

We have examined the decomposition at pH 7.4 of GSNO, generated in solution from glutathione and nitrous acid, in the presence of a low concentration of ascorbic acid and with added Cu^{2+} (1×10^{-4} mol dm^{-3}). Fig. 1 shows the resulting absorbance–time plots measured at 545 nm.‡ Trace (*a*) shows the small extent of decomposition (< 10% in 3 h) which occurs when no ascorbic acid is present. This probably represents the spontaneous thermal decomposition. This is in marked contrast to trace (*b*) which results from the same experiment with added ascorbic acid (2×10^{-4} mol dm^{-3}). Here, reaction goes to completion by a first order process with a half life of *ca.* 18 min.

This reaction is dramatically stopped, virtually completely (over the time scale examined), by the addition, at the start, of EDTA (1×10^{-3} mol dm^{-3}), as shown in trace (*c*). Further, when the experiment of trace (*b*) is repeated, and EDTA (1×10^{-3} mol dm^{-3}) is added after about one half life, then the reaction is stopped suddenly at this time point, as shown in trace (*d*). These experiments show clearly that the GSNO decomposition under these conditions *is* a metal ion promoted reaction, almost certainly a Cu^{+} catalysed reaction, in which the ascorbate ion is acting as a reducing agent for Cu^{2+}, in the same way as thiolate does in the earlier experiments,[1] and leading to disulfide formation. A similar reaction using *S*-nitrosocysteine (which is much faster than the reaction of GSNO), with added Cu^{2+} (5×10^{-6} mol dm^{-3}) and ascorbic acid (5×10^{-6} mol dm^{-3}), generated 86% NO as detected by a commercial NO electrode.

However at higher ascorbate concentrations the situation is somewhat different. For example, when the GSNO experiments are repeated with ascorbate at a ten-fold higher concentration (2×10^{-3} mol dm^{-3}), there is a slow first order decomposition, which is completely unaffected by the addition of EDTA (1×10^{-3} mol dm^{-3}) after roughly one half life. Furthermore the reaction seems to be quite general, since the decomposition of the more reactive *S*-nitrosopenicillamine occurs readily in the presence of EDTA, and the rate is clearly dependent on the [ascorbate], as shown in Fig. 2. When reactions are carried out under conditions of [ascorbate] >> [RSNO], then we find a good first order dependence on both reactants, for a number of RSNO structures. The products of this reaction at higher [ascorbate] are NO and the thiol. For example, in the reaction of *S*-nitrosocysteine with 0.1 mol dm^{-3} ascorbate, the thiol was detected by the Ellman procedure[8] in > 80% yield. This contrasts markedly with the copper-promoted reaction carried out at 1×10^{-5} mol dm^{-3} ascorbate, where the thiol

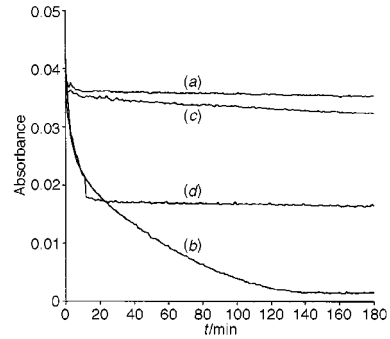

Fig. 1 Absorbance–time plots (measured at 545 nm) for the decomposition of GSNO (2×10^{-3} mol dm^{-3}) with added Cu^{2+} (1×10^{-4} mol dm^{-3}) at pH 7.4: (*a*) with no added ascorbic acid, but with added EDTA (1×10^{-3} mol dm^{-3}); (*b*) with added ascorbic acid (2×10^{-4} mol dm^{-3}); (*c*) as (*b*) but with added EDTA (1×10^{-3} mol dm^{-3}); (*d*) as (*b*) but with EDTA (1×10^{-3} mol dm^{-3}) added after 14 min

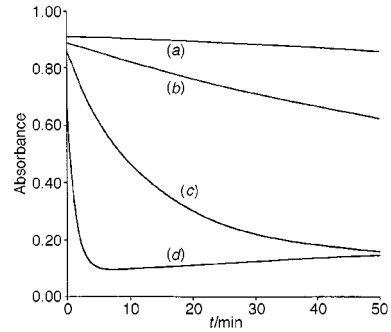

Fig. 2 Absorbance–time plots (measured at 340 nm) for the decomposition of *S*-nitrosopenicillamine (1×10^{-3} mol dm^{-3}) in the presence of EDTA (1×10^{-3} mol dm^{-3}) and (*a*) 0, (*b*) 1×10^{-4}, (*c*) 1×10^{-3} and (*d*) 1×10^{-2} mol dm^{-3} ascorbate

concentration in the product is < 5%. Similarly we were able to detect NO in high yield (78%) from the same reaction at high [ascorbate] (0.01 mol dm^{-3}) in the presence of EDTA (1×10^{-3} mol dm^{-3}) using the NO electrode. Similar results were obtained for the *S*-nitrosothiols derived from captopril, *N*-acetylpenicillamine, homocysteine and thioglycerol.

This reaction bears a close similarity to that recently reported for the reaction of ascorbate with an alkyl nitrite.[9] That reaction, also yielding NO, was studied at high pH (10–12), and was thought to involve initial attack at the nitroso nitrogen atom by the dianion of ascorbic acid. In our case, it is likely at pH 7.4 that the reactant is the monoanion of ascorbic acid, given that the two pK_a values of ascorbic acid are 4.3 and 11.8.

Nucleophilic attack at the nitroso nitrogen atom of *S*-nitrosothiols (and alkyl nitrites) is well-known for the thiolate ion [eqn. (4)], as the transnitrosation reaction, and has been

$$\text{RSNO (or RONO)} + \text{R}'\text{S}^- = \text{RS}^- \text{ (or RO}^-\text{)} + \text{R}'\text{SNO} \quad (4)$$

studied mechanistically.[10,11] More recently we have found[12] that other nucleophiles, such as amines, hydrazine, azide ion, and sulfite ion, will also act in this way; in each case the *S*-nitrosothiol acts as an electrophilic nitrosating species. *N*-Nitroso species, particularly *N*-nitrososulfonamides, have also been shown to be able to act as electrophilic nitrosating species towards a large range of nucleophiles.[13]

It is therefore not surprising that the ascorbate ion can also be nitrosated in a direct reaction with *S*-nitrosothiols. Nitrosation of ascorbic acid by nitrous acid is a well-known reaction which has been studied mechanistically.[14,15] Reaction is thought to involve *O*-nitrosation, followed by homolytic cleavage of the O–NO bond to give NO and a radical or radical ion species, which yields dehydroascorbic acid as the final product, by reaction with more nitrous acid. Alternatively, initial attack could (by analogy with nitrosation of some phenols[16]) occur at carbon, as an electrophilic addition to a very reactive alkene, followed by homolytic fission to give NO.

Whatever the detailed mechanism of NO release in these reactions with ascorbate, it is clear that there is a reaction pathway for NO formation from *S*-nitrosothiols which is independent of the presence of metal ions, particularly Cu^{2+}. This reaction is dominant at high [ascorbate], whereas at low [ascorbate], where the direct ascorbate reaction is too slow to be significant, the Cu^+-promoted reaction takes over. Presumably the copper reaction is suppressed anyway at high [ascorbate], by complexation and hence removal of Cu^{2+}, just as is the case for many of the Cu^+-promoted reactions where thiolate ion is the reducing agent.

These results show clearly that there are two reactions between ascorbate and *S*-nitrosothiols, and explain the observations[4,5] that GSNO can still undergo decomposition in the presence of ascorbate, even when Cu^{2+} ions are removed, so long as the [ascorbate] is high enough to ensure a reasonable rate of reaction.

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Notes and References

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‡ For the slower reactions we worked at 545 nm, rather than at the more usual wavelength of 340 nm (where there is a higher extinction coefficient), to avoid the increasing absorbance towards the end of the reactions in the 340 nm region, which occurs because of the decomposition reaction of the product dehydroascorbic acid (ref. 7).

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