Reaction of ascorbic acid with *S*-nitrosothiols: clear evidence for two distinct reaction pathways

Anthony J. Holmes and D. Lyn H. Williams

Department of Chemistry, Durham University, South Road, Durham, UK DH1 3LE.
E-mail address: D.L.H.Williams@durham.ac.uk

Received (in Cambridge, UK) 19th May 2000, Accepted 14th June 2000
Published on the Web 19th July 2000

Ascorbate reacts with *S*-nitrosothiols generally, in the pH range 3–13 by way of two distinct pathways, (a) at low [ascorbate], typically below ~1×10^{-4} mol dm^{-3} which leads to the formation of NO and the disulfide, and (b) at higher [ascorbate] when the products are the thiol and NO. Reaction (a) is Cu^{2+}-dependent, and is completely cut out in the presence of EDTA, whereas reaction (b) is totally independent of [Cu^{2+}] and takes place readily whether EDTA is present or not. For *S*-nitrosoglutathione (GSNO) the two reactions can be made quite separate, although for some reactants the two reactions overlap. In reaction (a), ascorbate acts as a reducing agent, generating Cu^{+} from Cu^{2+}, which in turn reacts with RSNO forming initially NO, Cu^{2+} and RS^{-}. The latter can then play the role of reducing agent for Cu^{2+}, leading to disulfide formation. Ascorbate will initiate reaction when the free thiolate has initially been reduced to a very low level by the synthesis of RSNO from a large excess of nitrous acid over the thiol. Reaction (b) is interpreted in terms of nucleophilic attack by ascorbate at the nitroso-nitrogen atom, leading to thiol and *O*-nitrosoascorbate which breaks up, by a free-radical pathway, to give dehydroascorbic acid and NO. A similar pathway is the accepted mechanism in the literature for the nitrosation of ascorbate by nitrous acid and alkyl nitrites. The rate constant for the Cu^{2+} -independent pathway increases sharply with pH and analysis of the variation of the rate constant with pH identifies a reaction pathway *via* both the mono- and di-anion forms of ascorbate, with the latter being the more reactive. As expected the entropy of activation is large and negative. Some aspects of structure–reactivity trends are discussed.

The chemistry of *S*-nitrosothiols RSNO continues to be a topic of major interest within the wider much publicised physiological roles of nitric oxide. *S*-Nitrosothiols are much more stable than is nitric oxide itself in the *in vivo* situation,[1] and since they generally show the same biological properties as does NO,[2] it has been suggested that NO is stored and transported around the body in the RSNO form.[3] Specific examples which have been detected *in vivo* and which are believed to be involved in this way include *S*-nitrosogluthathione (GSNO), *S*-nitrosoproteins (such as *S*-nitrosoalbumins), and more recently *S*-nitrosohaemoglobin,[4] for which a specific role in the control of blood pressure has been suggested. RSNO compounds are also being actively tested as potential NO-donors to make up for deficiencies of normal NO synthesis.

Nitric oxide release from RSNO can occur thermally, but is very slow for all RSNO at room temperature.[5] A number of misconceptions exist, particularly in the early biological literature, which unfortunately continue to be quoted. For example a very recent paper,[6] fails to recognise that in the absence of a Cu^{2+} ion chelator (see later), the thermal reaction is completely overwhelmed by the Cu^{2+}-catalysed reaction (even when Cu^{2+} is present at impurity low levels), and apart from claiming an erroneous order of reaction (which is not explained mechanistically), the rates are orders of magnitude in error. Decomposition can also take place photochemically (but this requires irradiation), and also in solution by a Cu^{2+}-catalysed reaction. The latter is by far the most important reaction in the absence of appropriate irradiation, and can occur at very low levels of Cu^{2+}. The reaction is actually brought about[7] by Cu^{+}, formed by reduction by RS^{-}, which is always present in low concentration when RSNO is synthesised from equimolar amounts of thiol and nitrous acid[8] (Scheme 1). It has also been shown[9] that Cu^{+} can also be generated by thiolate reduction of Cu^{2+} bound to peptides and proteins, thus allowing the possibility that this reaction could occur *in vivo*.

$$\mathrm{RSH + HNO_2 \rightleftharpoons RSNO + H_2O}$$
$$\mathrm{2Cu^{2+} + 2RS^- \longrightarrow 2Cu^+ + RSSR}$$
$$\mathrm{Cu^+ + RSNO \longrightarrow Cu^{2+} + RS^- + NO}$$

Scheme 1

Because in most cases, there is uncertainty about the [Cu^{2+}], and in all cases there is no knowledge of the exact value for the free [RS^{-}], together with the range of complications arising from complexation of Cu^{2+}, by either RSSR, RSH or maybe even by RSNO itself, which will be structure specific, *no reliance* can be put on any of the (large number of) values for the rate constants in the literature, for the decomposition of RSNO compounds which occur by the Cu^{2+}-catalysed process.

The other interesting reaction of *S*-nitrosothiols is that which occurs when they act as electrophilic nitrosating agents, *i.e.* when effective direct transfer of NO^{+} occurs to suitable nucleophilic sites (X), without the intermediate formation of any free nitrosating species (eqn. (1)). This reaction has been established

$$\mathrm{RSNO + X \rightleftharpoons RS^- + ONX^+} \qquad (1)$$

for direct transfer of NO in the NO^{+} sense for the transfer to thiolate ions ($X = RS^{-}$),[10] amines ($X = e.g.\ R_2NH$) and other nitrogen nucleophiles (*e.g.* $X = N_3^{-}$),[11] hydroperoxide ions ($X = HOO^{-}$)[12] and a range of other sulfur-centered nucleophiles (*e.g.* $X = SO_3^{2-}$).[13]

We considered it of some interest to establish what reactions, if any, occur between ascorbate and RSNO generally. In theory both reaction pathways mentioned above could exist, *i.e.* ascorbate could generate Cu^{+} by reduction, or ascorbate

DOI: 10.1039/b004028m

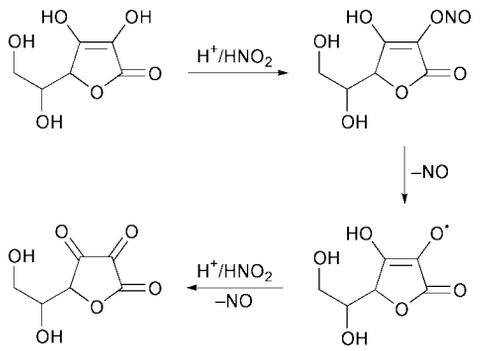

Fig. 1 The generally accepted sequence for the acid catalysed nitrosation of ascorbic acid by nitrous acid.

could act as a nucleophilic site for direct nitrosation by RSNO. These reactions could also have some relevance to the *in vivo* situation because of the presence and importance of ascorbic acid in the body. There is some literature evidence which suggests that maybe *both* reactions may occur. A few experiments noting the promotion of *S*-nitroso-*N*-acetylpenicillamine (SNAP) decomposition by ascorbate have been interpreted[14] in terms of ascorbate reduction of Cu^{2+}, whereas Japanese workers,[15,16] have showed that decomposition of *S*-nitrosoglutathione (GSNO) in the presence of ascorbate occurs slowly, even in the presence of a metal ion chelator. They also showed that the biological properties of GSNO are enhanced in the presence of ascorbate, suggesting that this is as a result of increased NO production. Other workers had noticed that the release of NO from GSNO and also from *S*-nitrosoalbumin in human blood plasma was also enhanced by the addition of some reducing agents, including ascorbate.[17]

The nitrosation of ascorbic acid in mildly acid solution by nitrous acid has been studied mechanistically.[18] It reacts with two moles of nitrous acid to give dehydroascorbic acid and two moles of nitric oxide (Fig. 1). Kinetic measurements show all of the expected characteristics of electrophilic nitrosation, and have identified the usual nitrosating agents, *i.e.* $H_2NO_2^+$ (or NO^+) at higher acidities and N_2O_3 at lower acidities, where, since more of the reactive ascorbate monoanion exists, the formation of N_2O_3 can be rate-limiting. Catalysis by halide and thiocyanate ions occurs as expected. Analysis of the reaction rate constants over a pH range shows that reaction in the acid range occurs *via* both the monoanion and the unionised forms, with, as expected, the rate constant for the former being the greater. In fact the value for the monoanion is very close to that found for the nitrosation of other anions such as azide, acetate and nitrite, and is believed to be at or close to the diffusion-controlled limit.[19] Under certain experimental conditions however, it has been shown that subsequent, inorganic reactions, notably the oxidation of the nitric oxide product can be rate-limiting. A recent study[20] throws some light on the decomposition pathway of the radicals generated by homolysis of the first formed *O*-nitrosoascorbic acid. Nitrosation of ascorbate by alkyl nitrites also occurs in neutral or alkaline solution. In the pH range 10–12, the reactive species is exclusively the ascorbate dianion.[21]

The reaction of nitrous acid with ascorbate is widely used as a source of nitric oxide, including its use as a primary standard for the NO-electrochemical probe for nitric oxide analysis in solution. Ascorbic acid has also been used as a trap for nitrous acid, particularly in the presence of secondary amines, when the formation of carcinogenic nitrosamines can be much reduced or effectively eliminated. There is evidence[22] which shows that tumour production in animals fed amines and nitrite is much reduced when ascorbic acid is also included. Ascorbic acid also acts as an efficient trap for nitrous acid, to ensure the irreversibility of the denitrosation of nitrosamines,[23] when

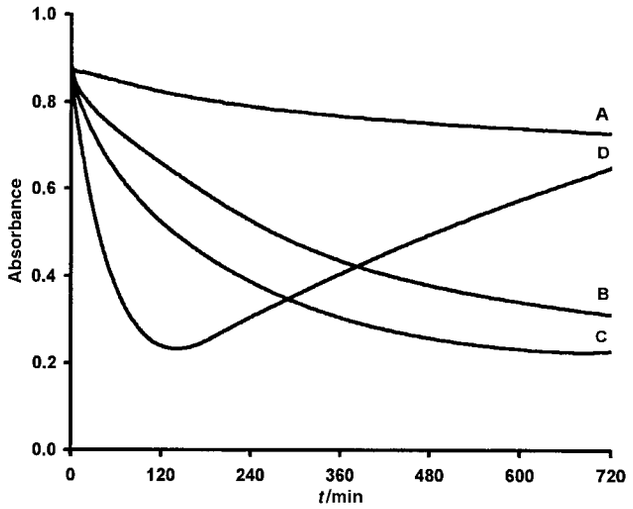

Fig. 2 Absorbance–time plots measured at 340 nm for the decomposition of *S*-nitrosoglutathione in the presence of ascorbic acid, A 1×10^{-5}, B 1×10^{-4}, C 1×10^{-3} and D 1×10^{-2} mol dm^{-3}.

often, small amounts of nitrosamines have to be removed from a range of household products, in order to meet the safety requirements.

This paper reports the results of an investigation into the reaction of ascorbic acid with a range of *S*-nitrosothiol structures. A preliminary account of some of our findings has already been published,[24] where evidence was presented that two separate reactions occur, one dominant at low [ascorbate] and the other at high [ascorbate].

Results and discussion

Decomposition of GSNO

Addition of ascorbate clearly promotes GSNO decomposition as shown in Fig. 2, which shows the absorbance due to GSNO at 340 nm (ε 895 dm^3 mol^{-1} cm^{-1}) against time, for different [ascorbate] for reaction at pH 7.4. Reaction is very slow at [ascorbate] = 1×10^{-5} mol dm^{-3} (trace A), and it probably does not proceed to completion, whereas the decomposition rate is increased with increasing [ascorbate]. Trace D shows an unusual feature in that the absorbance increases after about two hours. This probably arises from the decomposition of the expected product dehydroascorbic acid, a known reaction.[25] We confirmed this behaviour using an authentic sample of dehydroascorbic acid. To avoid this complication here and later with the more slowly reacting RSNO, we worked at the other absorbance maximum for GSNO at 545 nm (ε 20 dm^3 mol^{-1} cm^{-1}). These traces are necessarily more noisy, since the extinction coefficient is much lower at this wavelength. An example is shown in Fig. 3. In order to establish any part played by Cu^{2+}, experiments were performed in the presence of the well-known metal ion chelator EDTA. The results for GSNO were quite dramatic, in that at lower [ascorbate], $<1 \times 10^{-4}$ mol dm^{-3}, and at quite high [Cu^{2+}] ~ 1×10^{-4} mol dm^{-3}, where reaction is reasonably rapid (and proceeds to completion), reaction is virtually halted when EDTA is present, added either at the start of, or at some stage during the reaction.[24] However at much higher [ascorbate], up to 1×10^{-2} mol dm^{-3}, reaction takes place smoothly in the presence of EDTA, and leads to complete conversion. These results show a good first-order kinetic dependence on [ascorbate], *i.e.* the total stoichiometric concentration of ascorbate.

The NO electrode was used to monitor the effect of ascorbate on NO release from GSNO with added Cu^{2+}, even in the

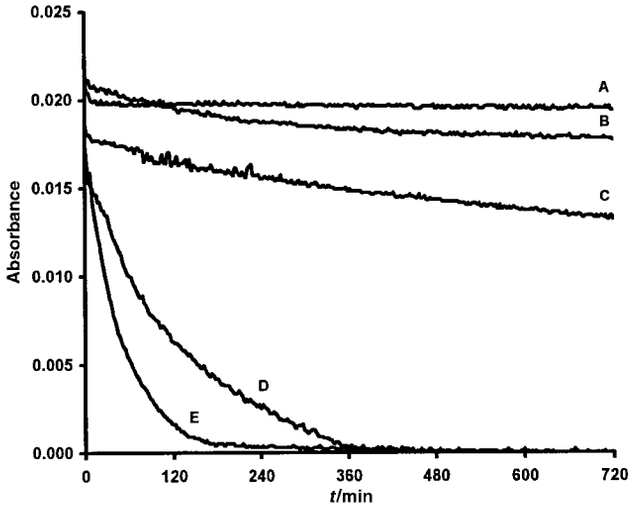

Fig. 3 Absorbance–time plots measured at 545 nm for the decomposition of *S*-nitrosoglutathione in the presence of ascorbic acid, A 0, B 1×10^{-5}, C 1×10^{-4}, D 1×10^{-3} and E 1×10^{-2} mol dm^{-3}.

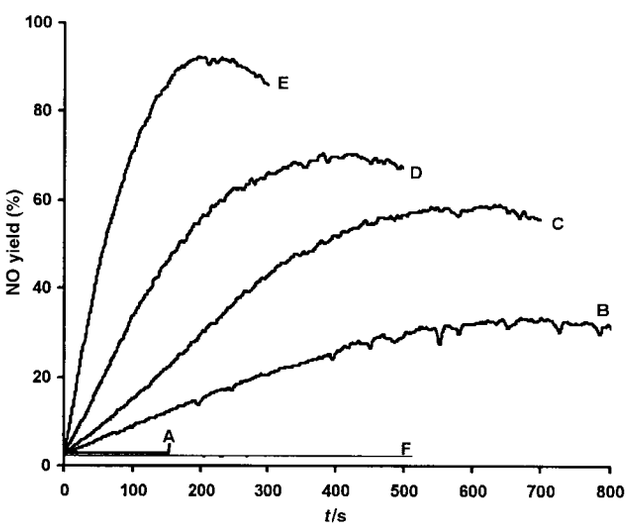

Fig. 4 % Yield NO from *S*-nitrosoglutathione containing Cu^{2+} (1×10^{-5} mol dm^{-3}) and the disulfide GSSG (1×10^{-4} mol dm^{-3}) with increasing concentrations of ascorbic acid, A 0, B 5.3×10^{-7}, C 1.0×10^{-6}, D 3.0×10^{-6}, E 1.3×10^{-5} and F 1.33×10^{-5} together with EDTA (1×10^{-4} mol dm^{-3}).

presence of added GSSG. In the absence of ascorbate, no reaction occurs, since Cu^{2+} is strongly complexed to GSSG,[26] but as the concentration of ascorbate is increased from 5×10^{-7} to 1.33×10^{-5} mol dm^{-3}, reaction occurs, progressively more rapidly and with increasing % conversion, until at the highest ascorbate concentration the yield of NO is virtually quantitative (see Fig. 4). Reaction is totally prevented in this ascorbate concentration range in the presence of EDTA (trace F), confirming that we are monitoring the Cu^{2+}-catalysed reaction under these conditions.

Decomposition of *S*-nitroso-*N*-acetylpenicillamine (SNAP)

The same general features were found for the reactions of SNAP as for GSNO, in that there are two reactions, (a) a Cu^{2+}-catalysed pathway, dominant at low [ascorbate], and (b) a Cu^{2+}-independent pathway, dominant at high [ascorbate]. A typical series of absorbance–time traces are shown in Fig. 5. There are however, significant differences between the reactions of GSNO and SNAP. The Cu^{2+} catalysed reaction is much more dominant for SNAP than it is for GSNO, for a reason which emerged recently.[27] This reaction is almost totally inhibited for

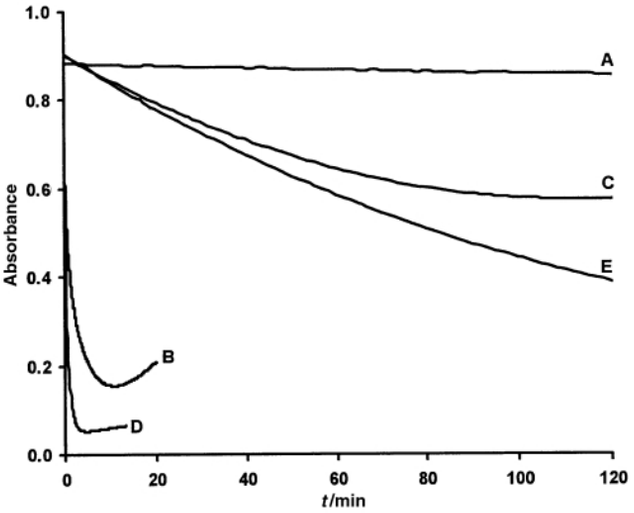

Fig. 5 Absorbance–time plots measured at 340 nm for the decomposition of *S*-nitroso-*N*-acetylpenicillamine in the presence of: A ascorbic acid (0.01 mol dm^{-3}) and EDTA (1×10^{-3} mol dm^{-3}), B ascorbic acid (0.01 mol dm^{-3}) and Cu^{2+} (1×10^{-5} mol dm^{-3}), C ascorbic acid (0.10 mol dm^{-3}) and EDTA (1×10^{-3}), D ascorbic acid (0.10 mol dm^{-3}) and Cu^{2+} (1×10^{-5} mol dm^{-3}) and E ascorbic acid (0.2 mol dm^{-3}) and EDTA (1×10^{-3} mol dm^{-3}).

GSNO and proceeds only to low conversion, when the [GSNO] is at millimolar concentration level, even with added $[Cu^{2+}] \sim 1 \times 10^{-5}$ mol dm^{-3}. This is due to the fact that Cu^{2+} is very effectively complexed by the disulfide product GSSG, almost certainly *via* the two glutamate residues. For GSNO the Cu^{2+}-promoted reaction can however still be made to occur in one of three ways: (a) if the concentration of GSNO is reduced to micromolar levels when much less of the Cu^{2+} will be complexed by GSSG, or (b) if the $[Cu^{2+}]$ is increased, or (c) if the $[RS^-]$ (or in principle any other reducing agent) is increased which will allow Cu^+ formation from the GSSG–Cu^{2+} complex. For SNAP the Cu^{2+}-independent pathway is slower than it is for GSNO, so we have used higher [ascorbate] for the SNAP reaction than for the GSNO reaction. The consequence here is that we see the Cu^{2+}-catalysed reaction (traces B and D) which is well separated from the Cu^{2+}-independent reaction (traces A, C and E), since under the experimental conditions used here, the former reaction is much faster than the latter. Similar behaviour was found with other RSNO species, all generated by thiol nitrosation, and used *in situ*.

When SNAP generated from *N*-acetylpenicillamine and nitrous acid with a 2:1 excess of nitrous acid is adjusted to pH 7.4, decomposition is virtually completely halted, even in the presence of some added Cu^{2+}, since the free thiolate concentration has been reduced to such a level that reduction of Cu^{2+} does not occur. Addition of low levels of ascorbate to this solution enables reaction to occur, at a rate which increases with the ascorbate concentration (see Fig. 6). This means that at least initially ascorbate can equally well bring about Cu^{2+} reduction, thus allowing decomposition of SNAP to occur. Since thiolate is released in this reaction, it may take over the role of reducing agent as reaction proceeds.

Product studies

A commercial electrochemical probe for NO was used to analyse the reaction product solutions, when reactions were carried out under nitrogen to limit the oxidation of NO. When the decomposition of *S*-nitrosocysteine (SNCys) (8.3×10^{-6} mol dm^{-3}) was carried out at pH 7.4, with added Cu^{2+} (5×10^{-6} mol dm^{-3}) and ascorbic acid (5×10^{-6} mol dm^{-3}), a value of 86% NO, based on the initial [SNCys] was measured in the final reaction solution. Similarly when the same reaction

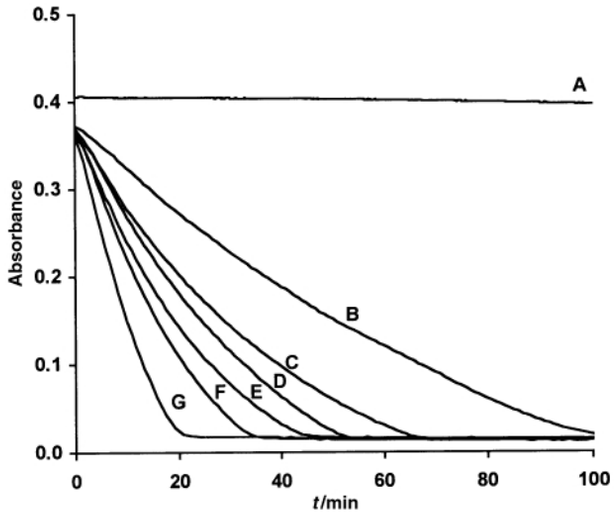

Fig. 6 Absorbance–time plots measured at 340 nm for the decomposition of *S*-nitroso-*N*-acetylpenicillamine (prepared with a two-fold excess of nitrous acid), in the presence of added Cu^{2+} (5×10^{-6} mol dm^{-3}): A with no added ascorbate, B, C, D, E, F and G, 5×10^{-6}, 8×10^{-6}, 1×10^{-5}, 1.5×10^{-5}, 2×10^{-5}, and 3×10^{-5} mol dm^{-3} respectively of added ascorbate.

was carried out with EDTA (1×10^{-3} mol dm^{-3}) and ascorbic acid (1×10^{-2} mol dm^{-3}), the yield of NO was found to be 78%. Given that a little oxidation and/or loss to the headspace probably occurred, we can reasonably assume that NO formation under both sets of experimental conditions is quantitative.

The decomposition of SNCys (1×10^{-3} mol dm^{-3}) was effected again at pH 7.4, (a) with added Cu^{2+} (1×10^{-5} mol dm^{-3}) and ascorbic acid (1×10^{-5} mol dm^{-3}), and (b) with EDTA (1×10^{-3} mol dm^{-3}) and ascorbic acid (1×10^{-1} mol dm^{-3}), and both final reaction solutions analysed for thiol (cysteine), using Ellman's method[28] calibrated with cysteine. For (a) we found <5%, and for (b) >80% thiol, from which we can deduce that the Cu^{2+}-independent reaction gives the thiol product probably quantitatively, whereas the Cu^{2+}-dependent reaction gives no thiol. An HPLC analysis of the product solution from the copper ion-dependent reaction (*i.e.* at very low [ascorbate]) for the reaction of GSNO showed clearly, as expected, that the disulfide was formed in >90% yield.

The Cu^{2+}-independent reaction

The stoichiometry of the reaction was investigated with SNCys, measuring the % decomposition spectrophotometrically at 545 nm, at constant [SNCys] and varying [ascorbate]. The results are shown in Table 1, and show clearly that complete decomposition of SNCys occurs (when the Cu^{2+}-promoted reaction is eliminated), only when the [SNCys]/[ascorbate] ratio is less than 2, showing that the reaction stoichiometry is 2 mol of *S*-nitrosothiol to 1 mol of ascorbate. This is the expected result, given the mechanism for the nitrosation of ascorbate by nitrous acid and alkyl nitrites, and is in full agreement with the result of Kashiba-Iwatsuki *et al.*[15] who also looked at the reaction of GSNO with ascorbate. Strangely a recent report[6] on the same reaction claimed, a 1:1 stoichiometry.

The kinetics of the reaction of ascorbate with a large range of *S*-nitrosothiols (all generated in solution and used *in situ*) were established, under conditions of [total ascorbate] ≫ [RSNO] and at pH 7.4, measuring the disappearance of an absorbance due to RSNO. All experiments gave good first order behaviour and plots of the measured first-order rate constant (k_{obs}) were linear in [total ascorbate] ($[H_2A]_T$), often with a small positive intercept at $[H_2A]_T = 0$, thus establishing

Table 1 % *S*-Nitrosocysteine (SNCys) decomposition at pH 7.4 in the presence of EDTA (1×10^{-3} mol dm^{-3}) as the [SNCys]/[Ascorbate] ratio is varied

[SNCys]/ mol dm^{-3}	[Ascorbate]/ mol dm^{-3}	[SNCys]/ [Ascorbate]	% SNCys Decomposition
0.010	2.0×10^{-3}	5.0	49
0.010	3.0×10^{-3}	3.3	72
0.010	4.0×10^{-3}	2.5	85
0.010	5.0×10^{-3}	2.0	94
0.010	7.0×10^{-3}	1.4	100
0.010	2.0×10^{-2}	0.5	97

Table 2 Values of k (eqn. (2)) for a range of RSNO

RSNO	$k/10^{-4}$ dm^3 mol^{-1} s^{-1}
S-Nitrosopenicillamine	14000 ± 140
S-Nitroso-*N*-acetylpenicillamine	32.2 ± 4.0
S-Nitrosocysteine	2540 ± 40
S-Nitroso-*N*-acetylcysteine	27.3 ± 1.1
S-Nitrosocysteamine	1460 ± 480
S-Nitroso-*N*-acetylcysteamine	42.6 ± 1.5
S-Nitrosoglutathione	150 ± 11
S-Nitrosohomocysteine	34.1 ± 1.7
S-Nitrosothioglycerol	15.9 ± 0.78

$$k_{obs} = k[H_2A]_T + k' \quad (2)$$

eqn. (2). Values of k were obtained for a large range of RSNO species, establishing the generality of the reaction. The small intercept (k') is taken to be the contribution to decomposition of RSNO by the thermal pathway. Some of the values are given in Table 2. The choice of RSNO was not meant as a mechanistic probe from a structure–reactivity analysis, but rather to represent the range of RSNO used in NO-donor experiments. Two features do however stand out, (a) the acetylation of a free amine group in *S*-nitrosopenicillamine, *S*-nitrosocysteine *etc.*, markedly reduces the reactivity. It is not immediately clear why this should be, but a possible explanation is that there is an electrostatic interaction between the $-NH_3^+$ (at pH 7.4 the amine group will mainly be in the protonated form) and one of the negatively charged oxygen atoms of the ascorbate dianion, involving some cyclic transition state (at pH 7.4, reaction primarily occurs *via* the dianion (see later)), and (b) there is a significant *gem*-dimethyl effect when the amino group is non-acetylated (*S*-nitrosopenicillamine *vs.* *S*-nitrosocysteine). This effect disappears when the amino group is acetylated (*S*-nitroso-*N*-acetylpenicillamine *vs.* *S*-nitroso-*N*-acetylcysteine), supporting the suggestion that there is no cyclic transition state when the amino group is acetylated. The reduced reactivity of *S*-nitrosohomocysteine compared with *S*-nitrosocysteine supports the suggestion that some cyclic transition state could be involved, which would be less favoured in the former, because of the presence of the extra methylene group.

Variation of rate constant with pH

The second-order rate constants k (eqn. (2)) for the reaction of *S*-nitrosocysteamine (2×10^{-3} mol dm^{-3}) with ascorbate (0.10 mol dm^{-3}) in the presence of EDTA (1×10^{-3} mol dm^{-3}) were obtained over the pH range 3–10.4. The values are given in Table 3. It is clear that the rate constants increase very sharply with pH above pH ~6. It is likely therefore that reaction occurs both *via* the mono- and di-anion of ascorbic acid, given that the two pK_a values are 4.25 and 11.75, since there is no levelling off of the rate constant after ~pH 5.5. It is also clear that the reactivity of the undissociated acid is negligible compared with that of the other two forms, since the rate constant at

Table 3 Values of k (eqn. (2)) for the reaction of *S*-nitrosocysteamine with ascorbic acid as a function of the pH

pH	$k/10^{-3}$ dm^3 mol^{-1} s^{-1}
3.0	0.27
3.0	0.20
3.3	0.28
3.5	0.33
4.2	0.89
4.5	1.26
4.5	1.28
5.0	1.90
5.2	2.21
5.5	3.68
5.8	7.50
7.4	119
9.5	669
10.4	1020

Table 4 Values of k (eqn. (2)) for the reaction of *S*-nitrosoglutathione with ascorbic acid over a wide pH range

pH	$k/10^{-3}$ dm^3 mol^{-1} s^{-1}
3.6	0.15
4.6	0.37
5.6	0.58
6.5	1.98
7.3	11.5
7.3	13.3
8.5	183
9.7	1020
10.1	2810
10.8	9830
11.5	56000
12.5	177000
13.7	323000

[a] The standard error in each k values is ~6%, and k is reproducible to ~ ± 7%.

pH < ~5 is negligibly small. We do not have enough data for the reaction of *S*-nitrosocysteamine to allow a detailed quantitative analysis, but have obtained many more points for the corresponding reaction of GSNO over a sufficiently wide pH range (3.6–13.7), given the two pK_a values of ascorbic acid. The results are in Table 4, and show qualitatively, as before, that reaction *via* the undissociated form H_2A is negligibly slow, and that we have to consider reaction pathways *via* both the monoanion HA^- and the dianion A^{2-}, eqns. (3) and (4). This leads to the expression given in eqn. (5) for the form of the

$$GSNO + HA^- \longrightarrow GS^- + DHA \qquad k_1 \qquad (3)$$

$$GSNO + A^{2-} \longrightarrow GS^- + DHA \qquad k_2 \qquad (4)$$

$$k = (k_1K_1[H^+] + k_2K_1K_2)/(K_1K_2 + K_1[H^+] + [H^+]^2) \qquad (5)$$

measured second order rate constant k (eqn. (2)). Here K_1 and K_2 are the two dissociation constants of ascorbic acid, and DHA is the product dehydroascorbic acid. Initially an estimated value for k_2 was taken to be 323 dm^3 mol^{-1} s^{-1}—the value of k at pH 13.7—and this was used in eqn. (5) in the pH range 3–9, where the value of k is sensitive to that of k_1, to obtain a crude value of k_1. These values were then used in connection with the Scientist computer package to obtain more reliable values. We found for GSNO, k_1 to be $6.3 \pm 0.1 \times 10^{-4}$ dm^3 mol^{-1} s^{-1} and k_2 to be 220 ± 20 dm^3 mol^{-1} s^{-1}. Calculated and experimental values of k over the full pH range are shown in the log k *vs.* pH plot in Fig. 7. The agreement is good, and

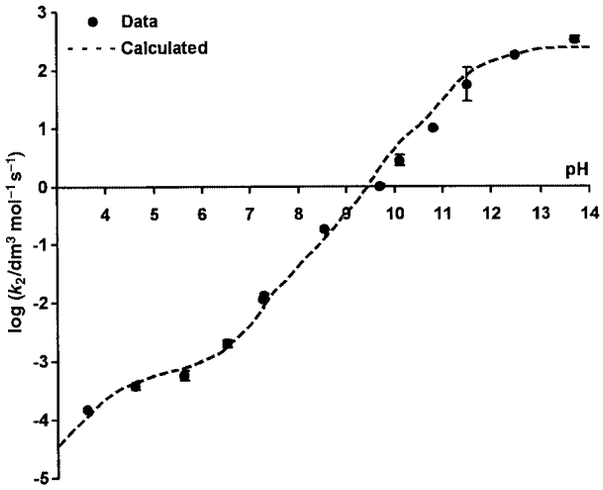

Fig. 7 The calculated curve (from eqn. (5)) and the experimental points for a plot of log k eqn. (2)) against pH for the reaction of *S*-nitrosoglutathione with ascorbic acid.

the expected features are present, *i.e.* the levelling off of log k above the second pK_a value, and the point of inflection where the levelling off above the first pK_a value overlaps with the increasing value of the rate constant as reaction *via* the dianion becomes important. A recent paper[6] has also investigated this reaction and come to the same conclusion *i.e.* that reaction occurs *via* both the mono- and di-anion forms of ascorbic acid. Their analysis gave values at 25 °C of $5.2 \pm 1.5 \times 10^{-3}$ and $1.22 \pm 0.04 \times 10^4$ dm^3 mol^{-1} s^{-1} for k_1 and k_2 respectively, which are not in good agreement with our values. We consider our values to be the more reliable, since we covered the appropriate pH range (3.6–13.7), and obtained a good correlation over the whole pH range whereas the other study only covered the range 5.3–8.0, where the values of the measured rate constants are very insensitive to the choice of k_2 values. Using our results it is clear that at the physiological pH of 7.4, reaction proceeds mostly (>90%) by way of reaction of the dianion of ascorbic acid. The reaction of alkyl nitrites with ascorbic acid has also been studied kinetically,[21] but only in the pH range 10–12, where as expected the dianion is the only reactive form.

The enthalpy and entropy of activation were measured for the reactions of *S*-nitrosopenicillamine and *S*-nitroso-1-amino-2-methylpropane-2-thiol with ascorbic acid at pH 7.4. Here ~95% of the reaction proceeds *via* the dianion, so no attempt was made to obtain separate values for the reactions of the mono- and di-anion. We obtained values of 68 ± 4 and 59 ± 3 kJ mol^{-1} for the overall enthalpies of activation and −21 ± 9 and −50 ± 9 J K^{-1} mol^{-1} for the entropies of activation. These are as expected for a bimolecular process.

Conclusions

We have established that *S*-nitrosothiols generally react with ascorbic acid in aqueous solution in two reactions. At low [ascorbate], the products are nitric oxide and the corresponding disulfide, whereas at higher [ascorbate] the products are again nitric oxide and the thiol from which the *S*-nitrosothiol is derived. The former reaction is Cu^{2+}-promoted and is completely halted by a metal ion chelator, whereas the latter is unaffected by Cu^{2+} and takes place in the presence of EDTA. All our results suggest that the Cu^{2+}-promoted reaction occurs by generation of Cu^+ by ascorbate reduction of Cu^{2+}, which then generates NO from RSNO, releasing RS^-, which can then take over the reduction role and lead to disulfide formation. The other reaction occurs with the mono- and di-anion forms

of ascorbic acid in a ratio which will be governed by the pH of the medium. We have interpreted this reaction in terms of electrophilic nitrosation by RSNO at one of the oxygen sites of the ascorbate ions, followed by free-radical breakdown of the *O*-nitrosated species to give NO and dehydroascorbic acid. This fits in with (a) the recent results demonstrating the nitrosating ability of RSNO towards a wide variety of nucleophilic substrates, and (b) with the accepted mechanism for the nitrosation of ascorbic acid by nitrous acid and also by alkyl nitrites. An alternative mechanism involving a one-electron transfer has recently been proposed,[6] which does not fit into this general pattern.

Experimental

All chemical reagents were purchased at the highest purity grade available and used as such. *S*-Nitrosothiols were all generated in acid solution from a 1:1 mixture of nitrous acid and the corresponding thiol, except for the experiments where the residual [thiol] was reduced to negligible levels, when a large excess of nitrous acid was used. The stock solution was neutralised to the appropriate pH using phthalate–HCl, phthalate–NaOH, phosphate–NaOH, tris–HCl, bicarbonate–NaOH buffers. Kinetic measurements were made following the disappearance of the RSNO absorptions at either 340, 400 or 545 nm, depending on whether there was any interference from the decomposition reaction of the product dehydroascorbic acid. Reaction conditions in the kinetic experiments for the copper-independent reaction were typically [RSNO] ~ 1×10^{-3} mol dm^{-3}, with the total stoichiometric [ascorbic acid] at least in 20-fold excess. Measurements were carried out in a conventional or stopped-flow UV–visible spectrophotometer, depending on the reaction rate. Data were collected and analysed using the computer software accompanying the spectrometer. Good first-order kinetics were found, and the first-order rate constant (k_{obs}) was reproducible to better than ±3%. Plots of k_{obs} against $[H_2A]_T$ were all linear with a very small positive intercept, and so values of k (eqn. (2)) were obtained from the slope. In the spectrophotometric product analyses and in the experiments carried out to establish the stoichiometry of the copper-independent reaction, a range of RSNO concentrations were employed.

Thiol concentration in the final reaction solution from the Cu^{2+}-independent reaction was determined by the Ellman procedure,[28] using a calibration graph generated from the appropriate thiol. Nitric oxide was determined using the commercial WPI NO electrode system which was calibrated with NO generated from ascorbic acid and nitrous acid. The disulfide was determined quantitatively for the reaction of GSNO in the copper ion-dependent reaction by reversed phase HPLC using a spherisorb ODS2 column with eluent comprising 90% pH 2.5 phosphate buffer and 10% methanol and a flow rate of 0.70 cm^3 min^{-1}. Under these conditions there was a clear separation between GSSG and the thiol GSH.

Acknowledgements

We thank the EPSRC for a research studentship to AJH and for a research grant for the purchase of a stopped-flow spectrophotometer, and Dr Darren Noble for discussions and assistance with the analyses.

References

1 E. A. Kowaluk and H. L. Fung, *J. Pharmacol. Exp. Ther.*, 1990, **255**, 1256.
2 J. S. Stamler, D. I. Simon, J. A. Osborne, M. E. Mullins, O. Jaraki, T. Michel, D. J. Singel and J. S. Loscalzo, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 444.
3 J. S. Stamler, O. Jaraki, J. Osborne, D. I. Simon, J. Keaney, J. Vita, D. Singel, C. R. Valeri and J. Loscalzo, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 7674.
4 L. Jia, C. Bonaventura, J. Bonaventura and J. S. Stamler, *Nature*, 1996, **380**, 221.
5 D. L. H. Williams, *Acc. Chem. Res.*, 1999, **32**, 869.
6 J. N. Smith and T. P. Dasgupta, *Nitric Oxide: Biol. Chem.*, 2000, **4**, 57.
7 A. P. Dicks, H. R. Swift, D. L. H. Williams, A. R. Butler, H. H. Al-Sa'doni and B. G. Cox, *J. Chem. Soc., Perkin Trans. 2*, 1996, 481.
8 P. H. Beloso and D. L. H. Williams, *Chem. Commun.*, 1997, 89.
9 A. P. Dicks and D. L. H. Williams, *Chem. Biol.*, 1996, **3**, 655.
10 D. J. Barnett, A. Rios and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1995, 1279.
11 A. P. Munro and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1999, 1989.
12 P. J. Coupe and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1999, 1057.
13 A. P. Munro and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 2000, in the press.
14 A. P. Dicks, P. H. Beloso and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1997, 1429.
15 M. Kashiba-Iwatsuki, M. Yamaguchi and M. Inoue, *FEBS Lett.*, 1996, **389**, 149.
16 M. Kashiba-Iwatsuki, K. Kitoh, E. Kasahara, H. Yu, M. Nisikawa, M. Matuso and M. Inoue, *J. Biochem.* (*Tokyo*), 1997, **122**, 1208.
17 G. Scorza, D. Pietraforte and M. Minetti, *Free Radical Biol. Med.*, 1977, **22**, 633.
18 C. A. Bunton, H. Dahn and L. Loewe, *Helv. Chim. Acta*, 1960, **43**, 303; C. A. Bunton, H. Dahn and L. Loewe, *Helv. Chim. Acta*, 1960, **43**, 317; C. A. Bunton, H. Dahn and L. Loewe, *Helv. Chim. Acta*, 1960, **43**, 320 and earlier papers.
19 J. H. Ridd, *Adv. Phys. Org. Chem.*, 1978, **16**, 1.
20 B. D. Beake, R. B. Moodie and D. Smith, *J. Chem. Soc., Perkin Trans. 2*, 1995, 1251.
21 J. R. Leis and A. Rios, *J. Chem. Soc., Chem. Commun.*, 1995, 169; J. R. Leis and A. Rios, *J. Chem. Soc., Perkin Trans. 2*, 1996, 857.
22 W. Lijinsky, *Chemistry and biology of N-nitroso compounds*, Cambridge University Press, 1992, p. 37.
23 S. S. Mirvish, L. Wallcave, M. Eagen and P. Shubik, *Science*, 1972, **177**, 65; D. L. H. Williams, *Food. Cosmet. Toxicol.*, 1978, **16**, 365.
24 A. J. Holmes and D. L. H. Williams, *Chem. Commun.*, 1998, 1711.
25 E. L. Hirst, E. G. V. Percival, R. W. Herbert, R. J. W. Reynolds and F. Smith, *J. Chem. Soc.*, 1933, 1270.
26 K. Varnagay, I. Sovago and H. Kozlowski, *Inorg. Chim. Acta*, 1988, **151**, 117.
27 D. R. Noble, H. R. Swift and D. L. H. Williams, *Chem. Commun.*, 1999, 2317.
28 P. W. Riddles, R. L. Blakeley and B. Zerner, *Anal. Biochem*, 1979, **94**, 75.