Nitric oxide release from *S*-nitrosoglutathione (GSNO)

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Received (in Cambridge, UK) 30th September 1999, Accepted 11th October 1999

In the presence of Cu^{2+} (10^{-5} M) very little NO is generated from GSNO at ~10^{-3} M at pH 7.4, whereas the reaction is quantitative at ~10^{-6} M; this is explicable in terms of the complexation of Cu^{2+} by the product GSSG.

Interest in the biological chemistry of nitric oxide (NO) continues to expand as its range of known biological functions increases. *S*-Nitrosothiols (RSNO) are currently believed to play an important part *in vivo* in the storage and transport of NO.[1] Additionally, RSNO species, particularly *S*-nitrosoglutathione (GSNO), have wide applicability as NO donors.

It has been something of a puzzle that very little NO appears to be generated from GSNO in solution at pH 7.4 when it is at a concentration of ~1×10^{-3} mol dm^{-3}, whereas this nitrosothiol has been, and continues to be, widely used as a NO donor in a variety of experiments, both *in vitro* and *in vivo*. Indeed GSNO has been used for this purpose medicinally.[2] Most of the experiments relating to mechanistic studies of this reaction have followed the disappearance, spectrophotometrically, of the band at ~340 nm, characteristic of all RSNO species. Because of the relatively low extinction coefficients at this wavelength (~10^3 mol dm^{-3} cm^{-1}), suitable concentrations for conventional spectrophotometers are in the range 10^{-3}–10^{-4} mol dm^{-3}. The reaction mechanism has been shown[3] to involve [eqns. (1) and (2)] reaction with Cu^+ generated by reduction of Cu^{2+} by thiolate ion; the former is

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

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

often present as an impurity at low concentration levels, and the latter is always present at low concentration, because of the slight reversibility of RSNO formation from nitrous acid and a thiol.[4] We,[5] and others,[6] have shown that even at these millimolar concentrations, reaction can, however, be made quantitative by the addition of further amounts of Cu^{2+} or thiolate ion. For example the half-life for effectively quantitative decomposition of GSNO (5×10^{-4} mol dm^{-3}) containing added Cu^{2+} (5×10^{-5} mol dm^{-3}) and glutathione (GSH) (5×10^{-5} mol dm^{-3}) is ~15 min. Addition of the metal ion chelator EDTA stops the reaction completely.

We now report results from the direct measurement of NO produced from GSNO over a range of initial concentrations of GSNO (3×10^{-6} to 4×10^{-4} mol dm^{-3}), all at pH 7.4, and in the presence of added Cu^{2+} (1×10^{-5} mol dm^{-3}). Measurements were made using a commercial NO probe (WPI ISO-NO Mark II), which was calibrated using NO generated from nitrous acid and ascorbate. The results are quite dramatic, and are presented in Fig. 1 as the percentage yield NO as a function of time, generated from the different GSNO concentrations. At the lowest concentrations studied it is clear that the yield of NO is essentially quantitative, whereas as the [GSNO] is increased this yield falls, until at 4×10^{-4} mol dm^{-3} the yield of NO is only ~5%.† The last trace confirms the result (~5% decomposition) obtained spectrophotometrically at this concentration. Again no reaction occurred when EDTA was added. A similar result was obtained without added Cu^{2+}, relying on the impurity Cu^{2+} levels in the water/buffer components, but reaction rates were somewhat less.

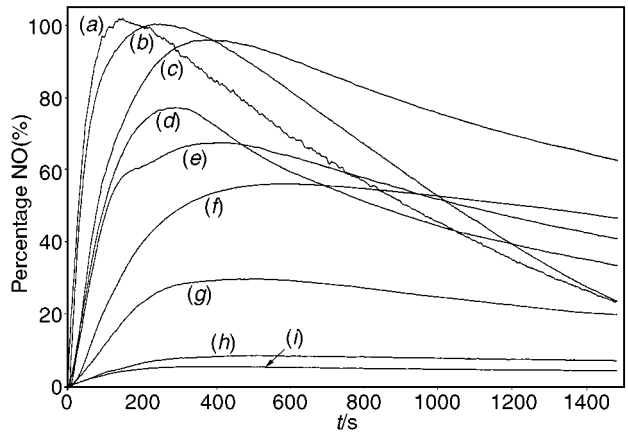


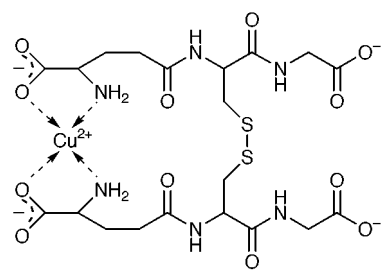
Fig. 1 The percentage NO generated from GSNO in the presence of added Cu^{2+} (1×10^{-5} mol dm^{-3}) as a function of the initial [GSNO]: (*a*) 3.0×10^{-6}, (*b*) 6.2×10^{-6}, (*c*) 1.2×10^{-5}, (*d*) 2.0×10^{-5}, (*e*) 2.3×10^{-5}, (*f*) 3.1×10^{-5}, (*g*) 7.4×10^{-5}, (*h*) 2.2×10^{-4} and (*i*) 3.7×10^{-4} mol dm^{-3}.

An explanation of these unusual results can be obtained if we consider the possibility that the organic product, the oxidised form of glutathione (GSSG), itself complexes the Cu^{2+} ions. Such complexes are well-known,[7] and have been identified spectrally. The suggested structure for the 1:1 complex with GSSG (absorption maxima at 250 and 625 nm) is given as **1**, but at higher [Cu^{2+}] a 2:1 complex has also been isolated and its crystal structure established.[8] The characteristic shoulder at 250 nm could be observed in the final spectrum from GSNO decomposition in our experiments.

To test this idea more directly we carried out the reaction of GSNO (3×10^{-6} mol dm^{-3}) in the presence of increasing amounts of added GSSG in the range 1×10^{-5} to 2×10^{-4} mol dm^{-3}). The results are given in Fig. 2. It is clear that, over this range, the percentage yield of NO decreases progressively from ~90 to ~10%.

Exactly the same pattern (not shown) was found for the reaction studied spectrophotometrically, *i.e.* at higher [GSNO], with added Cu^{2+} and GSH, when again addition of GSSG progressively stopped the reaction at incomplete conversion.

This explains our present set of results. The product GSSG is in effect acting as a metal ion chelator (for Cu^{2+} in this case), preventing reaction from occurring as the copper is removed from solution. It is to be expected that this effect is more marked



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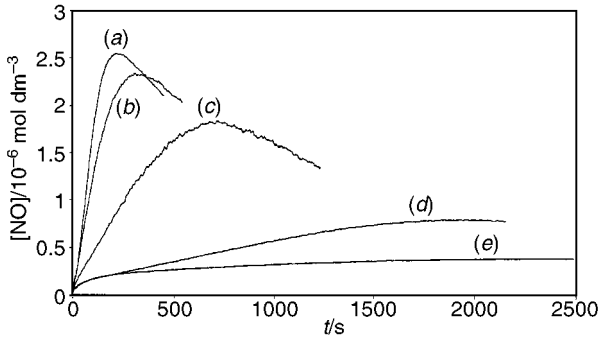

Fig. 2 The percentage NO generated from GSNO (3.1×10^{-6} mol dm^{-3}) in the presence of added Cu^{2+} (1×10^{-5} mol dm^{-3}) as a function of added GSSG: (*a*) 0, (*b*) 1.0×10^{-5}, (*c*) 2.0×10^{-5}, (*d*) 5.0×10^{-5} and (*e*) 2.0×10^{-4} mol dm^{-3}.

at the higher [GSNO], since more GSSG is then generated. Obviously the addition of more Cu^{2+} under these conditions will help promote reaction, as will the addition of GSH, since we propose that thiol/thiolate will be able to generate Cu^{+} from the GSSG–Cu^{2+} complex. We have tested this by addition of GSH to a solution of GSSG–Cu^{2+}, when the low extinction absorbance peak at 625 nm disappeared, and when the specific Cu^{+} chelator neocuproine[9] was added, the characteristic absorbance at 453 nm was immediately formed, corresponding to 82% conversion to Cu^{+}. So added GSH not only generates Cu^{+} from Cu^{2+} to start off the reaction, but also can generate Cu^{+} from the product GSSG–Cu^{2+} complex, allowing reaction to go on to completion. In the absence of added GSH however, when its concentration is very low, the inequality [GSSG] > [GSH] soon applies in the early stages of the reaction, and Cu^{+} cannot be retrieved from the GSSG–Cu^{2+} complex and reaction effectively ceases.

Higher concentrations of added GSH (1×10^{-4} to 1×10^{-3} mol dm^{-3}) inhibit NO formation from GSNO, probably since GSH–Cu^{+} complexes[10] are formed. Much higher thiolate concentrations lead to a totally new reaction where the main nitrogen product is ammonia and not NO.[11]

It has also been suggested[6] that the Cu^{+}-promoted decomposition of GSNO does not involve reoxidation of Cu^{+}, since no complex formation occurred when the specific Cu^{2+} chelator cuprizone was added. We found that Cu^{2+} is complexed more strongly to GSSG than it is to cuprizone, since when Cu^{2+} was added to equal concentrations of cuprizone and GSSG, no cuprizone complex is formed. However, we also find that cuprizone does stop the reaction of *S*-nitroso-*N*-acetylpenicillamine (SNAP), added either at the start or after ~60% reaction. In this case the disulfide does not have an inhibiting effect on the reaction.

We have looked briefly at the decomposition characteristics of *S*-nitrosocysteine (SNC). In contrast to the behaviour of GSNO, SNC decomposes quantitatively to give NO and cystine, even at the higher millimolar concentrations. When we examine the decomposition as a function of initial [SNC], we find that there is no great change, either in the rate of reaction, or in the NO yield over the range 3×10^{-6} to 1×10^{-3} mol dm^{-3}. The absence of the glutamyl residue here does not allow complex formation as in **1**.

It is probable the unreactivity of *S*-nitrosocaptopril at millimolar concentrations arises from the Cu^{2+}-complexing ability of the product disulfide since it contains a similar arrangement of atoms as in the glutamate residue in glutathione. This hypothesis remains to be tested.

A necessary consequence of our present results is that no structure–reactivity conclusions can be drawn from the rate constants (measured over a range of [Cu^{2+}]) of different RSNO structures, *when they have been measured at the millimolar concentration level*. This includes a series of rate constants reported earlier by us.[12] We no longer need to invoke the requirement of bidentate coordination of Cu^{+} with the nitroso group and a free amino group. GSNO and SNC have comparable decomposition reactivities at micromolar concentrations. The apparent greater reactivity of SNC at millimolar concentrations is not due, as previously thought, to the enhanced reactivity of SNC (because of the possibility of strong bidentate coordination of Cu^{+}), but rather due to the much reduced reactivity of GSNO due to the strong chelation of Cu^{2+} by the product GSSG. This effect was also found to be a major factor in the decomposition characteristics of a novel *S*-nitroso sugar derivative.[13]

We thank the EPSRC for a Research Grant which supported this work.

Notes and references

† Ideally the NO traces should level off when reaction is effectively over. The falling away must represent loss of NO either by oxidation or some other reaction.

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Communication 9/07891F