Generation of nitric oxide from *S*-nitrosothiols using protein-bound Cu^{2+} sources

Andrew P Dicks and D Lyn H Williams

Background: We have recently shown that *S*-nitrosothiols (RSNOs) decompose in aqueous buffer to give nitric oxide, an important signalling molecule, and the corresponding disulphides. This occurs by reaction with Cu^{+} generated from Cu^{2+} (supplied as hydrated Cu^{2+}) by thiolate reduction. To establish whether these reactions are feasible *in vivo*, we set out to determine whether Cu^{2+} bound to an amino acid, a tripeptide or to human serum albumin (HSA) could serve as a Cu^{+} source for generation of NO from *S*-nitrosothiols.

Results: Experiments with Cu^{2+} bound to the tripeptide Gly-Gly-His or to two histidine molecules or to HSA showed that Cu^{+} was released (and trapped with neocuproine) when the copper source was treated with a thiol at pH 7.4. RSNO decomposition was achieved with all three copper sources, although not as rapidly as with added hydrated Cu^{2+}. Decomposition was also catalyzed by ceruloplasmin.

Conclusions: These results show clearly that amino-acid- and protein-bound Cu^{2+} can be reduced by thiolate ion to Cu^{+}, which will generate NO from RSNO species, thus providing a realistic model for these reactions *in vivo*.

Address: Department of Chemistry, Durham University, Durham DH1 3LE, UK.

Correspondence: D Lyn H Williams
e-mail: D.L.H.Williams@durham.ac.uk

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Introduction

Interest in the chemistry and biology of nitric oxide (NO) is now intense, following the series of discoveries since 1986, indicating that it is synthesized *in vivo* from L-arginine and has a role in many physiological processes [1–4]. *S*-Nitrosothiols (RSNOs), sometimes called thionitrites, but henceforth referred to as nitrosothiols, are important as potential therapeutic drugs for the *in vivo* delivery of NO and as possible intermediates in the *in vivo* chemistry and transport of NO; *S*-nitrosoglutathione (GSNO) and other nitrosothiols have been detected *in vivo* [5]. The physical and chemical properties of nitrosothiols have been reported [6,7].

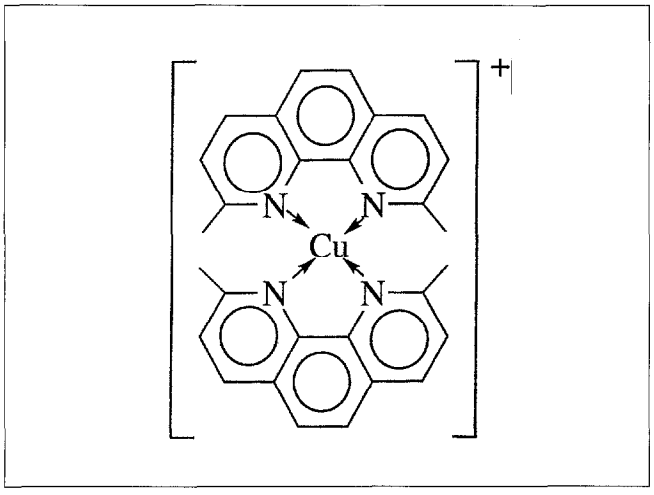
Glyceryl trinitrate (GTN) is by far the most successful and widely used NO-releasing drug, and a mechanism for the reaction has been proposed [8]. GTN has been used clinically for over 100 years for the treatment of angina and problems of the blood circulation system. It suffers, however, from certain drawbacks — it is rather short acting and some patients soon develop a tolerance to it [9]. Thus, there has been much effort directed at the design of alternative NO-releasing drugs, and nitrosothiols are attractive possibilities, since it is known that they liberate NO in solution. The biological properties of nitrosothiols are akin to those of NO itself; they induce vasodilation [10–12] and prevent platelet aggregation [13,14]. There is some debate as to whether the action of nitrosothiols depends on their ability to release NO, or whether they act without such prior decomposition. Evidence has been presented in favour of both views [15,16]. Whatever the mechanism may be, there is no doubting their clinical efficacy; GSNO has been shown in clinical trials to be effective in the prevention of platelet aggregation after coronary angioplasty [17] and also for the treatment of a form of pre-eclampsia [18].

Nitrosothiols decompose both thermally and photochemically initially according to equation (1), although NO will react further in the presence of oxygen.

$$2\mathrm{RSNO} \xrightarrow{\Delta \text{ or } h\nu} \mathrm{RSSR} + 2\mathrm{NO} \qquad (1)$$

The photochemical decomposition of GSNO, when irradiated at 340 nm or 545 nm, has been recently described [19]. There are also many reports in the literature of the decomposition of nitrosothiols in solution, typically at pH 7.4, when the initial products are the same as in equation (1), but in the presence of oxygen the NO is converted quantitatively to NO_2^-. We have recently established the main mechanistic features of the decomposition of nitrosothiols in aqueous buffers. Reaction is brought about by Cu^{2+} but not significantly by any other metal ions [20]. Even at very low concentrations, where Cu^{2+} is present as an impurity in the water or buffer (typically 1×10^{-6} M), reaction can occur. The rate of reaction is clearly dependent on the concentration of Cu^{2+}; it is thus not surprising that a range of rate constants has been reported in the literature, given the likely spread of

Figure 1



Structure of the Cu^+ complex with neocuproine.

adventitious Cu^{2+}-concentration values. No reaction occurs when Cu^{2+} is removed from solution with EDTA, even for the most reactive compounds (e.g., *S*-nitrosocysteine). More recently, it has become clear that the true reagent in these reactions is in fact Cu^+, generated by reduction of Cu^{2+} by thiolate ion [21] (eq. 2), which is a well known reaction [22,23]. Thiolate ion could be present in these solutions as a derivative of any thiol impurities in RSNO samples (given that they are synthesized by nitrosation of thiols [7]) or by hydrolysis of nitrosothiols (eq. 3), a reaction that has been demonstrated independently of the metal reduction reaction.

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

$$RSNO + 2OH^- = RS^- + NO_2^- + H_2O \quad (3)$$

Mayer and colleagues [24] have also recently shown that GSNO decomposition is brought about by Cu^+. Rates of reaction are of course dependent on the concentration RS^- (as well as on Cu^{2+} concentration), which explains further why values reported in the literature, obtained before this mechanistic interpretation was presented, are so variable and are generally worthless. There is a large structure–reactivity dependence, and analysis of the results shows that the greatest reactivity occurs when Cu^+ can coordinate bidentately with two nucleophilic sites within the nitrosothiol.

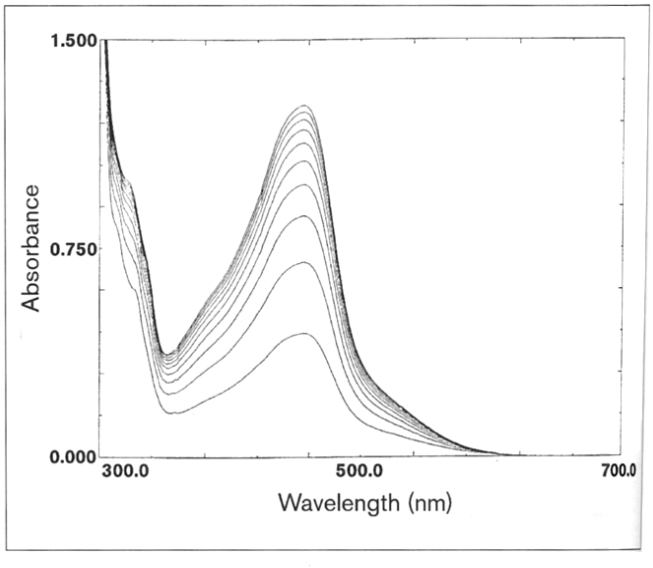
All of these results were obtained with hydrated Cu^{2+} ion as the source of copper; this ion may be present as an impurity or added as a Cu(II) salt. Although there is almost 0.1 g of copper per 75 kg human body weight, very little of it is present as hydrated Cu^{2+}. If we are to consider this copper-mediated reaction as a potential source of NO *in vivo*, it is clearly important to establish whether protein-bound sources of Cu^{2+}, such as those present in the body, can release Cu^+ and thus effect the decomposition of nitrosothiols.

Results

We chose to examine the possible reactions of *S*-nitrosothiols with Cu^{2+} bound to three different molecules: to two molecules of histidine (2HIS-Cu^{2+}), to the tripeptide Gly-Gly-His (GGH-Cu^{2+}) or to human serum albumin (HSA), bound as the mono-(HSA-Cu^{2+}) complex. It has been argued [25] that GGH-Cu^{2+} is a good model for *in vivo* binding of copper, which has an association constant of 8×10^{15} dm^3 mol^{-1}. Initially we chose to work with *S*-nitrosocysteine (SNC), because of the importance of cysteine *in vivo*. SNC is not stable in its pure form, but solutions can readily be prepared by mixing equimolar amounts of cysteine and sodium nitrite in mildly acidic aqueous solutions. Such stock solutions (at ~1×10^{-2} M) were sufficiently stable to use, and fresh stock solutions were prepared for each group of experiments. We also carried out similar experiments with two other *S*-nitrosothiols, to establish the generality of copper-mediated decomposition for the production of nitric oxide.

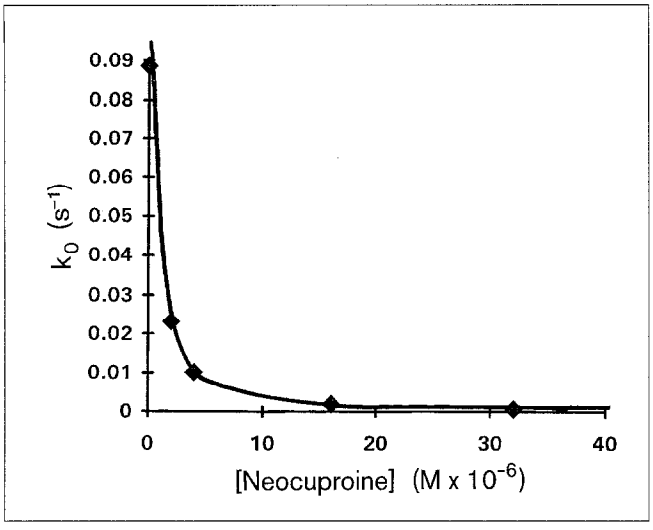
We first established whether thiolate ion would reduce these bound forms of Cu^{2+} to Cu^+. This seemed to be the case qualitatively, since the addition of an equimolar amount of cysteine to a solution of GGH-Cu^{2+} (2×10^{-3} M) at pH 7.4 caused an immediate and substantial reduction

Figure 2



Treatment of GGH-Cu^{2+} with the thiol *N*-acetyl penicillamine results in release of Cu^+. The reaction is carried out in the presence of the Cu^+ chelator neocuproine, and generation of Cu^+ is detected by an increase in the absorbance at 453 nm, which is characteristic of the Cu^+ chelate of neocuproine.

Figure 3



SNC decomposes in the presence of GGH-Cu^{2+} with a first-order rate constant. In the presence of excess neocuproine the reaction is inhibited due to chelation of free Cu^{+}.

of the absorbance at 527 nm, which is due to the Cu^{2+} complex (data not shown). When GGH-Cu^{2+} (2 x 10^{-4} M) was treated with an equivalent amount of a thiol in the presence of the specific Cu^{+} chelator neocuproine (4 x 10^{-4} M) [26], there was a quantitative and rapid build up of an absorbance at 453 nm which is characteristic of the Cu^{+}-chelate with the structure shown in Figure 1. The increasing absorbance for scans taken every minute is plotted in Figure 2, which shows the generation of the Cu^{+} complex from the reduction of GGH-Cu^{2+} by the thiol *N*-acetyl penicillamine. A very similar plot was obtained when cysteine was used as the reducing thiol. The same behaviour was also observed when HSA-Cu^{2+} was treated with either of these thiols. We obtained significant yields of the Cu^{+} chelate (68 % of the theoretical maximum from GGH-Cu^{2+}, 52 % from 2HIS-Cu^{2+} and 40 % from HSA-Cu^{2+}) for all three sources of bound Cu^{2+}. The yields are based on the known extinction coefficient of the Cu^{+}–neocuproine complex at 453 nm; treatment of free hydrated Cu^{2+} with reducing thiols resulted in 80 % reduction to Cu^{+}.

Decomposition of SNC occurred in the presence of these Cu^{2+} complexes. Addition of increasing amounts of neocuproine, however, progressively decreased the rate constant for the reaction, and decomposition was virtually halted at high neocuproine concentration (see Fig. 3). Exactly the same behaviour has been reported by us [21] and others [24] for the reactions in the presence of hydrated Cu^{2+}.

To compare the reactivities of the various complexes to that of hydrated Cu^{2+}, we have examined the rate of decomposition of RSNO in the presence of each species (Fig. 4). The bound forms of Cu^{2+} clearly bring about decomposition of nitrosothiols, but not as effectively as does the free hydrated Cu^{2+} ion itself. We obtained first-order rate constants in all cases as a function of the concentration of added copper complex. Figure 5 shows the relevant data for the decomposition reaction of SNC in the presence of added Cu^{2+}, which establish that the rate is described by equation 4. The measured first-order rate constant k_0 is given by equation 5 in which k^1 (the intercept of plots of k_0 vs copper-complex concentration) represents the reaction due to free hydrated Cu^{2+}, adventitiously present in the water/buffer components.

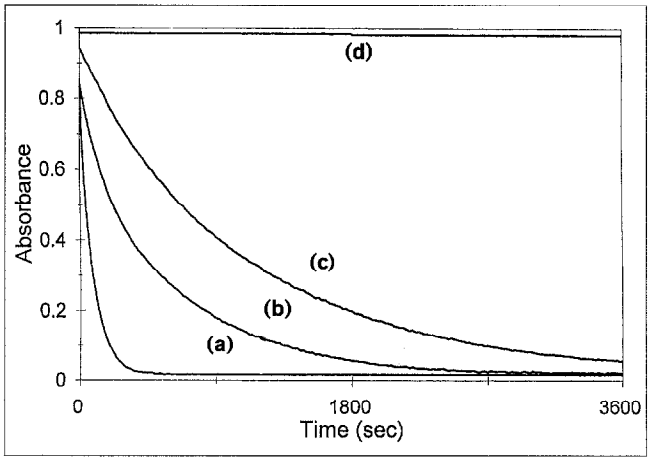
$$\text{Rate} = k\,[\text{copper complex}]\,[\text{RSNO}] + k^1\,[\text{RSNO}] \quad (4)$$

$$k_0 = k\,[\text{copper complex}] + k^1 \quad (5)$$

Similar plots were obtained for reactions in the presence of 2HIS-Cu^{2+},GGH-Cu^{2+} and HSA-Cu^{2+}. The slope of the line gives the value of k, and those values for the reactions with all the sources of Cu^{2+} studied are shown in Table 1. Similar results were obtained with two other nitrosothiols, *S*-nitroso-*N*-acetyl penicillamine (SNAP) and *S*-nitroso(2-mercaptopropionyl)glycine; it is thus likely that these reactions are general for all *S*-nitrosothiols.

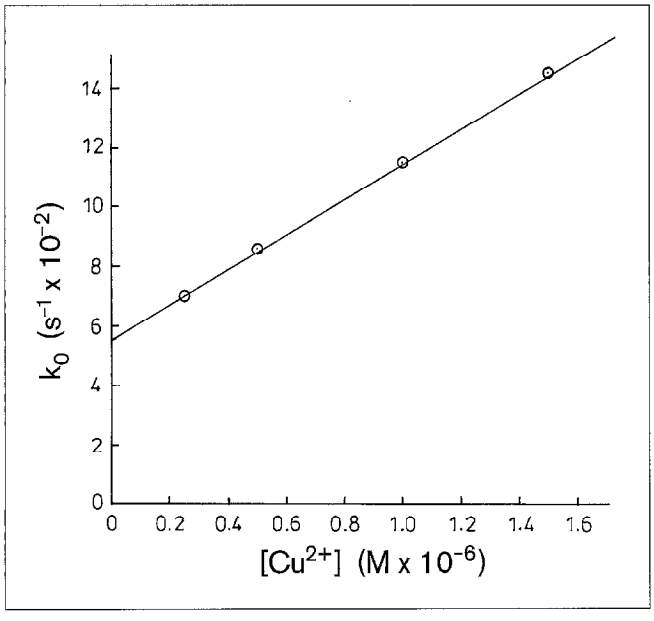
We examined whether the glyoprotein ceruloplasmin could serve as a copper source for the decomposition of *S*-nitrosothiols, since most of the total copper in blood plasma is complexed to this protein. Over the concentration range of 1–5 x 10^{-6} M ceruloplasmin, we found a linear dependence of the rate constant for SNC decomposition on the ceruloplasmin concentration (data not shown).

Figure 4



Different copper sources catalyze the decomposition of SNAP at different rates. **(a)** Cu^{2+} (1 x 10^{-5} M), **(b)** GGH-Cu^{2+} (1 x 10^{-5} M), **(c)** HSA-Cu^{2+} (1 x 10^{-5} M), **(d)** HSA (1 x 10^{-5} M).

Figure 5



The decomposition reaction of SNC in the presence of added Cu^{2+} has a first-order rate constant. The values of k_0 are shown as a function of Cu^{2+} concentration.

Discussion

We have shown here that Cu^+ can be generated from GGH-Cu^{2+}, 2HIS-Cu^{2+} and HSA-Cu^{2+} by treatment with a reducing thiol. The generation of Cu^+ was detected by the disappearance of the Cu^{2+} absorbance at 527 nm and the concurrent increase in the absorbance at 453 nm, when the reaction was carried out in the presence of neocuproine. A similar reduction of bound Cu^{2+} has been observed when ceruloplasmin [27], the blue copper oxidase of vertebrate plasma, is treated with cysteine. All of the copper complexes studied catalyzed the decomposition of nitrosothiols, releasing nitric oxide. In the reaction with SNAP, the reactivity order of the copper sources tested was: free hydrated Cu^{2+}> GGH-Cu^{2+}> HSA-Cu^{2+} (Fig. 4). HSA on its own seems to inhibit the reaction by complexation of the very small amount of Cu^{2+} present in the system, generating a concentration of HSA-Cu^{2+} of a much lower order of magnitude than those used in these studies. The Cu^+ chelator neocuproine progressively reduces the rate of SNC decomposition initiated by GGH-Cu^{2+}.

Table 1

Values of k (eq. 5) for the decomposition of SNC using various sources of Cu^{2+}.

Copper source	k ($dm^3\ mol^{-1}\ s^{-1}$)
Free hydrated Cu^{2+}	60 000
2HIS-Cu^{2+}	58 000
GGH-Cu^{2+}	11 600
HSA-Cu^{2+}	14 600

The reactivity order of the various copper sources with SNC was: free hydrated Cu^{2+}> 2HIS-Cu^{2+}> HSA-Cu^{2+}> GGH-Cu^{2+} (Table 1). All of these reaction rates are crucially dependent upon the concentration of RS^-, either present initially or generated by hydrolysis of RSNO. In this case all of the experiments were carried out using solutions of SNC prepared by nitrosation of cysteine. As expected, none of the bound forms of Cu^{2+} is as reactive as is the free hydrated Cu^{2+}; nevertheless, all show significant reactivity. We were also able to detect catalysis of SNC decomposition by ceruloplasmin, suggesting strongly that Cu^+ can be released from that source by thiolate reduction,which then can bring about nitric oxide release by decomposition of SNC.

Significance

Our experiments clearly show that Cu^{2+} bound to an amino acid, peptide or protein is accessible to reduction to Cu^+ by cysteine and other thiols in aqueous solution at pH 7.4. Cu^+ generated in this way is able to bring about the decomposition of *S*-nitrosothiols to yield nitric oxide in exactly the same way as does Cu^+ generated by reduction of free hydrated Cu^{2+}. This means that we can propose a realistic pathway for the formation of nitric oxide *in vivo* from *S*-nitrosothiols using copper sources present in the body. This pathway could be important mechanistically in providing an explanation for the role of *S*-nitrosothiols (which many people believe are important in the biology of nitric oxide) in the possible storage and delivery of nitric oxide *in vivo*. These results also provide a mechanism for nitric oxide generation from *S*-nitrosothiols administered therapeutically, which may lead to the widespread development of *S*-nitrosothiols as nitric oxide-releasing drugs.

Materials and methods

All of the nitrosothiols used in this work have been previously described [20,21]. Histidine, GGH, HSA and neocuproine were all commercial samples used without further purification, and the copper complexes were prepared by the addition of an equivalent amount of Cu^{2+}. Measurements were all made by conventional UV spectrophotometry and rate constants obtained by noting the disappearance of the absorbance due to the nitrosothiol at 340 nm and using conventional software packages.

Cu^+ formation

Typical experimental conditions were [GGH-Cu^{2+}] = [cysteine] = 2×10^{-4} M, [neocuproine] = 1×10^{-3} M; final absorbance at 453 nm = 1.10, corresponding to a Cu^+ yield of 68 %. Each measurement was carried out three times and the standard error was ~2 %.

Kinetic experiments

Stock solution of SNC was prepared from cysteine and sodium nitrite (both at about 1×10^{-2} M), and aliquots were added to a phosphate buffer at pH 7.4, containing the copper complex. The concentration of

the nitrosothiol was about 1 x 10^{-3} M at the start of the reaction, which was followed in a conventional spectrophotometer, noting the disappearance of the absorbance at 350 nm due to the nitrosothiol. The results were collected and analyzed using standard kinetics software packages. Reactions were first order and were reproducible to within ~3 %. Reactions with *S*-nitroso(2-mercaptopropionyl)glycine were carried out in the same way, whereas the experiments with SNAP involved making a standard solution of SNAP from the stable solid [28].

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