Mechanism of S-Nitrosothiol Formation and Degradation Mediated by Copper Ions*

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Experimental evidence is presented supporting a mechanism of *S*-nitrosothiol formation and degradation mediated by copper ions using bovine serum albumin, human hemoglobin and glutathione as models. We found that Cu^{2+}, but not Fe^{3+}, induces in the presence of NO a fast *S*-nitrosation of bovine serum albumin and human hemoglobin, and the reaction is prevented by thiol blocking reagents. During the reaction, Cu^+ is accumulated and accounts for destabilization of the *S*-nitrosothiol formed. In contrast, glutathione rapidly dimerizes in the presence of Cu^{2+}, the reaction competing with *S*-nitrosation and therefore preventing the formation of *S*-nitrosoglutathione. We have combined the presented role of Cu^{2+} in *S*-nitrosothiol formation with the known destabilizing effect of Cu^+, providing a unique simple picture where the redox state of copper determines either the NO release from *S*-nitrosothiols or the NO scavenging by thiol groups. The reactions described are fast, efficient, and may occur at micromolar concentration of all reactants. We propose that the mechanism presented may provide a general method for *in vitro S*-nitrosation.

S-Nitrosothiols (RS-NOs)[1] have a variety of biological activities, which are mostly attributed to their ability to release NO (1–3). RS-NOs are not only synthesized and administered clinically (2) but are also produced endogenously. Stamler *et al.* (4) reported that human plasma contains ~7 μM RS-NOs, mostly as *S*-nitroso-albumin, a level unexpectedly high as the basal cellular NO level is in the low nanomolar range (5, 6). Thus, RS-NOs are considered as NO pools buffering the level of NO, which may be targeted at different sites (7). RS-NOs are also reported to be involved in the trans-*S*-nitrosation of proteins by transferring the NO^+ moiety (8, 9), a process suggested to be a reversible post-translational modification regulating the activity of enzymes and receptors (3, 10, 11).

[1] The abbreviations used are: RS-NO, *S*-nitrosothiol; BSA, bovine serum albumin; BSA-SNO, *S*-nitroso-BSA; GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSSG, dimeric glutathione; Hb, human hemoglobin; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; NEM, *N*-ethylmaleimide; PMB, *p*-hydroxymercuribenzoic acid.

The degradation of RS-NOs depends on many factors including light, pH, metal ions and the presence of reductants (*e.g.* ascorbate or thiols) (12–16); RS-NOs are quite stable in pure buffer solutions (hours; Ref. 17), but they decompose rapidly (within seconds) upon irradiation with visible light or by transition metal catalysis (18). Therefore, their stability and reactivity in biological systems can hardly be predicted. RS-NO formation is even less understood than degradation. At neutral pH, NO does not react directly with glutathione (GSH) to form *S*-nitrosoglutathione (GSNO), since only a slow redox reaction forming N_2O and dimeric glutathione (GSSG) occurs ($k' = 4.8 \times 10^{-4} \, s^{-1}$ at 5 mM GSH; Ref. 19). Under aerobic conditions the auto-oxidation of NO generates N_2O_3 ($k \sim 6 \times 10^6 \, M^{-2} \, s^{-1}$; Ref. 20), which is able to *S*-nitrosate thiols. N_2O_3 reacts with both thiols and water, the two reactions proceeding at $k = 0.1 - 1 \times 10^5 \, M^{-1} \, s^{-1}$ (21), for low molecular weight thiols, and $k \sim 30 \, M^{-1} \, s^{-1}$ (21), respectively. It is worth noticing that the reaction with water efficiently competes with direct thiol nitrosation by N_2O_3, due to the large molar excess of water over thiols. NAD^+ substituting oxygen for the electron acceptor can also accelerate the reaction of NO with thiols (22). Several authors also suggested that *S*-nitrosation of thiols occurs by reaction with nitrosonium ions (NO^+) formed either via metal-catalyzed oxidation of NO or via dinitrosyl-iron-cysteine complexes (8, 21, 23, 24); efficiency and physiological relevance of these reactions remain unclear.

In this study we have examined by spectroscopic and amperometric techniques the interaction of NO and thiols in the presence of cupric and ferric ions. Experiments have been carried out using the small tripeptide GSH (low millimolar amounts in the cell), bovine serum albumin (BSA, which is the most abundant plasma protein), and human hemoglobin (Hb). BSA and GSH both bear only one reduced cysteine per molecule (Cys-34 in BSA; Refs. 25 and 26), but, as shown below, they display in the presence of Cu^{2+} a very different reactivity with NO. Hb has been reported to undergo *S*-nitrosation, the reaction occurring at the level of Cys-β93 (27). We found that Cu^{2+}, but not Fe^{3+}, catalyzes the rapid *S*-nitrosation of BSA with a stoichiometry of ~1 SNO/BSA, and of Hb with a stoichiometry dependent on the derivative used. GSH showed under similar conditions no reaction with NO, probably because of fast thiol dimerization. In contrast to other reports (8, 21, 23, 24), our evidence does not indicate a role for NO^+ in Cu^{2+}-induced *S*-nitrosation. As this Cu^{2+}-mediated reaction is fast, selective for thiols, and efficient, it may be relevant for RS-NO formation *in vitro*.

EXPERIMENTAL PROCEDURES

GSH was obtained from Roche Molecular Biochemicals; $HgCl_2$, $Cu(II)SO_4$, and Cu(I)Cl from Merck; DTNB, EDTA, NEM, PMB, neocuproine, and BSA (catalog no. A-2153) from Sigma Aldrich. Hb was purified according to Ref. 28. When necessary Hb was treated with thiol blocking reagents: 10-fold excess Hg^{2+} or 3–5-fold excess PMB over Hb tetramer. Solutions of BSA and GSH were used fresh in water, and the concentration of thiols was measured by the Ellman assay (29); GSH
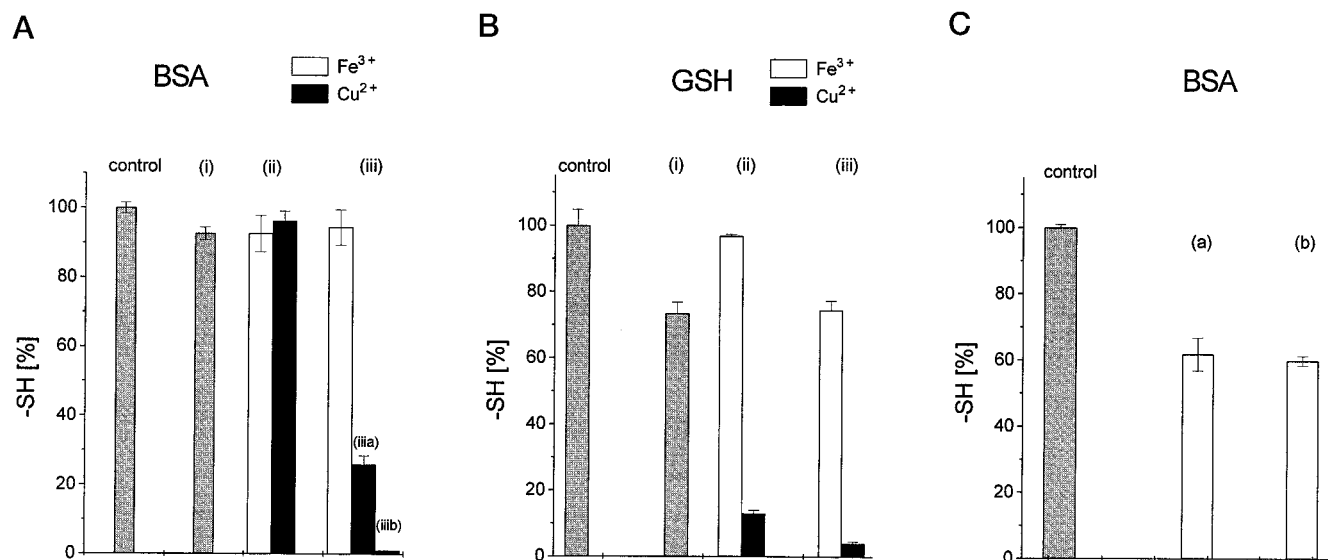
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FIG. 1. **Spectroscopic determination of free thiols: the effect of Cu^{2+}/Fe^{3+} and NO.** 7 μM BSA (*A*) or 7 μM GSH (*B*) were incubated aerobically for 3 min in 0.1 M phosphate buffer, pH 8.0, with (*i*) 60 μM NO, (*ii*) 35 μM metal ions, and (*iii*) 60 μM NO and 35 μM metal ions (the BSA measurements were also carried out in the absence (*iiia*) and presence (*iiib*) of 1 μM neocuproine). Then 1 mM EDTA and 100 μM DTNB was added and thiol concentration calculated. *C*, 11 μM BSA was incubated for 3 min aerobically (*a*) and anaerobically (*b*) with 45 μM Cu^{2+} and 12 μM NO, and thiol concentration was measured with DTNB. Control, thiol concentration of GSH or BSA without pretreatment of NO and Cu^{2+}.

had 1.0 ± 0.03 and BSA 0.53 ± 0.02 -SH/molecule. The understoichiometric concentration of free thiols in BSA is in agreement with previous reports (14) and probably due to mixed disulfides (30, 31). All BSA concentrations are therefore given with respect to free thiol concentrations, whereas concentration of Hb refers to the tetramer content. NO was purchased from Air Liquide (Paris, France) and purged from higher nitrogen oxides by passage trough a water-alkaline (KOH) trap. Stock solutions of NO were prepared by equilibrating degassed water with the purged NO gas at 1 atm; this yields 2.1 ± 0.1 mM NO at 20 °C (32), as also determined by spectrophotometric titration of reduced cytochrome *c* oxidase (33). Solutions of 10 mM Cu(I)Cl were always freshly prepared by dissolving 4 mg of CuCl in 4 ml of degassed 50 mM HCl and protecting the solution from light with aluminum foil. All procedures and measurements were carried out at 20 °C.

Spectroscopic measurements were performed using a Jasco V550 spectrophotometer (light path: 1 cm).

Measurement of Thiol Concentration—Cu^{2+} and/or NO were added to air-equilibrated buffer (0.1 M phosphate buffer, pH 8.0) containing either BSA or GSH. After 3 min, 1 mM EDTA was added to complex free Cu^{2+}, followed by the addition of 100 μM DTNB. EDTA was required, since thionitrobenzoic acid, the colored species that is released upon reaction of thiols with DTNB, is oxidized by Cu^{2+}. The absorption at 412 nm was measured after stable color formation (1–3 min) and, after subtracting the optical contribution of DTNB, thiol concentration was calculated using the extinction coefficient $\Delta\epsilon = 13.6$ mM^{-1} cm^{-1} (experimentally determined and in agreement with Ref. 29). Similar experiments have been performed using degassed buffer in a gas-tight cuvette.

Spectroscopic Measurement of BSA-SNO—An air-equilibrated solution of BSA in 0.1 M Hepes buffer, pH 7.5, was incubated for ~3 min with CuSO$_4$ and NO. Then 1 mM EDTA was added and a spectrum recorded. By subtracting the spectral contribution of Cu^{2+}, determined by performing a parallel experiment in the absence of NO, the absorption change at 340 nm revealed the amount of BSA-SNO formed (19). Finally, to measure the concentration of unreacted thiols, 100 μM DTNB was added and the absorbance change at 412 nm was determined after subtracting the optical contribution of DTNB.

NO Detection—NO was measured amperometrically using a Clark-type NO electrode (ISO-NO, World Precision Instruments, Sarasota, FL) connected to a 5.0-ml gas-tight glass vial sealed with a rubber septum. The electrode was calibrated with subsequent additions of NO-saturated water, and, after sample injection, the NO concentration in the electrode chamber was recorded continuously under constant stirring, at room temperature. Buffer was 0.1 M Hepes, pH 7.5.

RESULTS

Amperometric and spectroscopic experiments have been performed to explore the complex interaction of metal ions, NO,

thiols, and RS-NOs. The amperometric method is specific for NO (with no response to RS-NOs, nitrite, or NO$^+$), whereas the disappearance of free thiols and the formation of RS-NOs were followed spectroscopically. These experiments, carried out in parallel, provide complementary information, since the formation of RS-NOs implies the disappearance of both NO and free thiols.

Spectroscopic Thiol Determination—To investigate a possible role of Cu^{2+} in the formation of RS-NOs, GSH and BSA were aerobically mixed with: (i) NO, (ii) Cu^{2+}, or (iii) NO plus Cu^{2+}. Following incubation, the concentration of free thiols was determined photometrically by the Ellman reaction. The results in Fig. 1*A* show that, when NO and Cu^{2+} were added together, the free thiols of BSA decreased to 26 ± 3% (*n* = 3), whereas each reactant alone had essentially no effect (93 ± 2% and 96 ± 3% thiols, *n* = 3). Complete disappearance of free thiols was observed in the presence of 1 μM neocuproine (Fig. 1*A*, *iiib*), an efficient Cu^+ chelator (18), suggesting that cuprous ions are formed during the reaction of Cu^{2+} with BSA; since Cu^+ is known to cause degradation of RS-NOs (12, 18), its removal drains the equilibrium toward the complete formation of BSA-SNO.

Incubation of GSH with excess NO (for 3 min) had a small effect on the thiol concentration (−30%), while in contrast to BSA, free thiol concentration remarkably decreased (to 13 ± 1%) upon addition of Cu^{2+} alone (see Fig. 1*B*). In the presence of both NO and Cu^{2+}, thiols almost completely disappeared from solution.

Similar experiments carried out with ferric instead of cupric ions showed no disappearance of thiols using either BSA or GSH both with and without NO (Fig. 1, *A* and *B*). To assess a possible role of O_2 in the *S*-nitrosation of thiols (*e.g.* through formation of N_2O_3), similar experiments have been performed also under anaerobic conditions; to make the system sensitive to a putative effect of O_2, in these experiments NO was in slight excess over BSA. Under these conditions, ~40% of thiols disappeared within 3 min of incubation, regardless of the presence or absence of O_2 (Fig. 1*C*).

NO-electrode Measurements—When followed amperometrically, the NO concentration did not change by addition of either BSA or Cu^{2+} alone (Fig. 2, *A* and *B*). On the other hand, the
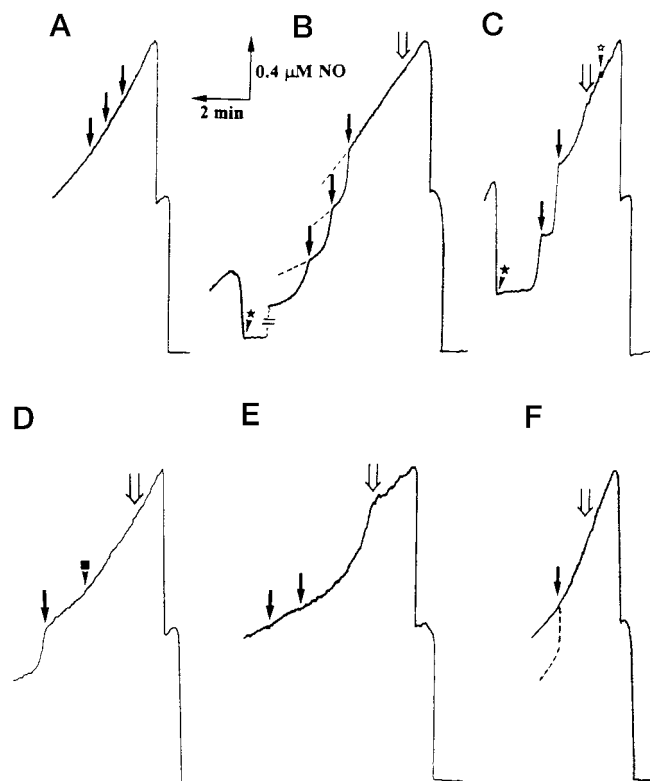
Fig. 2. **Amperometric measurements of the effect of Cu^{2+}, Fe^{3+}, BSA and GSH on NO.** To 5 ml of air-equilibrated buffer were added in sequence 2×1.0 μM NO and the following: *A*, 3×0.34 μM BSA ($\downarrow$) in the presence of 0.2 mM EDTA; *B*, 10 μM Cu^{2+} ($\Downarrow$), 3×0.34 μM BSA ($\downarrow$) and, after 10 min, 10 μM Cu^+ ($\star$); *C*, 1 μM neocuproine ($\star$), and 10 μM Cu^{2+} ($\Downarrow$), followed by 2×0.34 μM BSA ($\downarrow$) and 10 μM Cu^+ ($\star$); *D*, 10 μM Cu^{2+} ($\Downarrow$), 0.34 μM NEM pretreated BSA ($\blacksquare$) and 0.34 μM BSA ($\downarrow$); *E*, 10 μM Fe^{3+} ($\Downarrow$), followed by 2×0.34 μM BSA ($\downarrow$); *F*, 10 μM Cu^{2+} ($\Downarrow$) and 0.34 μM GSH ($\downarrow$). Reactants were added to the electrode chamber, and the NO concentration was measured continuously. *Dotted line* in *F* is reported for comparison and taken from *B*, corresponding to the decrease of NO when 0.34 μM BSA was added instead of GSH.

addition of BSA to a solution of both NO and Cu^{2+} was associated with disappearance of NO (Fig. 2*B*); the reaction was fast (seconds), being limited by the response time of the electrode (34), and yielded a stoichiometry of $\sim$0.7 NO/BSA. Interestingly addition of 10 μM Cu^+ released nearly all the NO uptaken by BSA (Fig. 2*B*); this finding provides direct evidence for the formation of BSA-SNO, as Cu^+ induces *S*-nitrosothiol decomposition with the release of NO (12, 18). The same experiment was performed in the presence of small amounts (1 μM) of neocuproine, chelating Cu^+ (presumably formed in the reaction of BSA with Cu^{2+}, see "Discussion"); under these conditions (Fig. 2*C*) upon addition of BSA, 1 NO/BSA disappeared from solution and the reaction was completely reversed upon addition of excess (over neocuproine) of Cu^+. Experiments performed in the presence of higher concentrations of neocuproine (>10 μM) led, after addition of Cu^{2+}, to a slight but significant disappearance of NO from solution independent of BSA (data not shown). This finding can probably be explained by the formation of a Cu^{2+}-neocuproine complex with high affinity for NO. To minimize this side reaction, which can still be observed in Fig. 2*C* (following addition of Cu^{2+}), neocuproine concentration was fixed at 1 μM. Amperometric measurements have been carried out with either air-equilibrated or N_2-equilibrated buffer; despite a clear increase of the spontaneous O_2-dependent disappearance of NO observed under aerobic conditions, the extent of NO disappearance caused by BSA addition was similar in both cases (data not shown). The latter

result further suggests that O_2 has no effect on the reaction of *S*-nitrosation of BSA (see also Fig. 1*C*).

To assess the involvement of thiol groups in the reaction of BSA and NO, the protein (0.36 mM) was preincubated with excess of either $HgCl_2$ (3 mM) or NEM (10 mM). Upon addition of derivatized BSA to the reaction chamber containing NO and Cu^{2+}, no disappearance of NO was observed (Fig. 2*D*). Moreover, addition of BSA to a solution containing NO and Fe^{3+} instead of Cu^{2+} did not result in a depletion of NO (Fig. 2*E*); only some NO disappearance after the addition of Fe^{3+} (independent of BSA) was observed. Therefore, consistent with the spectroscopic results, a catalytic activity of Fe^{3+} in BSA-SNO formation can be excluded. Consistently with the spectroscopic results (Fig. 1*B*), addition of GSH to a solution of NO and Cu^{2+} showed no significant disappearance of NO (Fig. 2*F*).

To test whether other proteins, apart from BSA, can be *S*-nitrosated through the proposed Cu^{2+}-mediated pathway, *S*-nitrosation of Hb was investigated by the amperometric NO assay just described. As shown in Fig. 3, when Hb either in the deoxy- or oxy-state was anaerobically added to the NO solution, a fast NO disappearance was observed corresponding to NO binding to the hemes in a 1:1 stoichiometry (independent of Hb treatment with thiol blocking reagents). Upon addition of Cu^{2+}, a further rapid disappearance of NO was detected, which was attributed to RS-NO formation, being prevented by treating Hb with thiol blocking reagents (Fig. 3, *B* and *D*) and reversed by addition of Cu^+ (Fig. 3, *A* and *C*). The measured SNO/Hb-tetramer stoichiometry was 1.0–1.2 using deoxy-Hb and 1.4–2.2 using oxy-Hb. The different yield can be tentatively rationalized, considering that the reactivity of Cys-β93 can change depending on the ligation state of Hb (27).

Spectroscopic Determination of S-Nitrosothiols—BSA-SNO formed during incubation of BSA with Cu^{2+} and NO was determined spectroscopically (Fig. 4). RS-NOs are characterized by a weak absorbance band with a maximum at 340 nm (19). Owing to the low extinction coefficient of BSA-SNO, all concentrations were increased by 1–2 orders of magnitude. Incubation of BSA at different concentrations (0–100 μM) with constant amounts of NO and Cu^{2+} showed an absorbance increase at 340 nm (Fig. 4*A*), which was not observed in the absence of Cu^{2+}. The linear regression of the data in Fig. 4*B* allowed us to calculate an extinction coefficient for BSA-SNO at 340 nm: $\epsilon = 0.77$ mM^{-1} cm^{-1}, in reasonable agreement with the published $\epsilon = 0.87$ mM^{-1} cm^{-1} at 334 nm (35) and very close to $\epsilon = 0.76$ mM^{-1} cm^{-1} for GSNO (19). When 1 mM EDTA was added to the solution containing BSA and Cu^{2+} before addition of NO, BSA-SNO formation was fully prevented (data not shown).

Effect of NO and Copper Concentration on BSA-SNO Formation—BSA was aerobically incubated with variable amounts of NO (0–200 μM) in the presence of Cu^{2+}. Then, excess EDTA was added to complex free cupric ions and both the formation of BSA-SNO and the concentration of free thiols (after the addition of 100 μM DTNB) were spectroscopically measured. As shown in Fig. 5, upon increasing the NO concentration, the fraction of free cysteine of BSA decreases while the BSA-SNO concentration increases. The reaction is very efficient since, even at a 1:1 NO-to-BSA ratio, $\approx$70% of the reaction has occurred.

Fig. 6 (*data set a*) reports the concentration of free thiols measured following the aerobic addition of BSA to constant amount of NO and varying the concentration of Cu^{2+} (0–70 μM). As shown in the figure, up to a Cu^{2+}-to-BSA ratio of 1:1, essentially no disappearance of free cysteines was observed; the further increase of Cu^{2+} led to a linear decrease of free cysteines up to 35–40% of the initial level. Further addition of Cu^{2+} was ineffective even up to a Cu^{2+}-to-BSA ratio of 10:1.
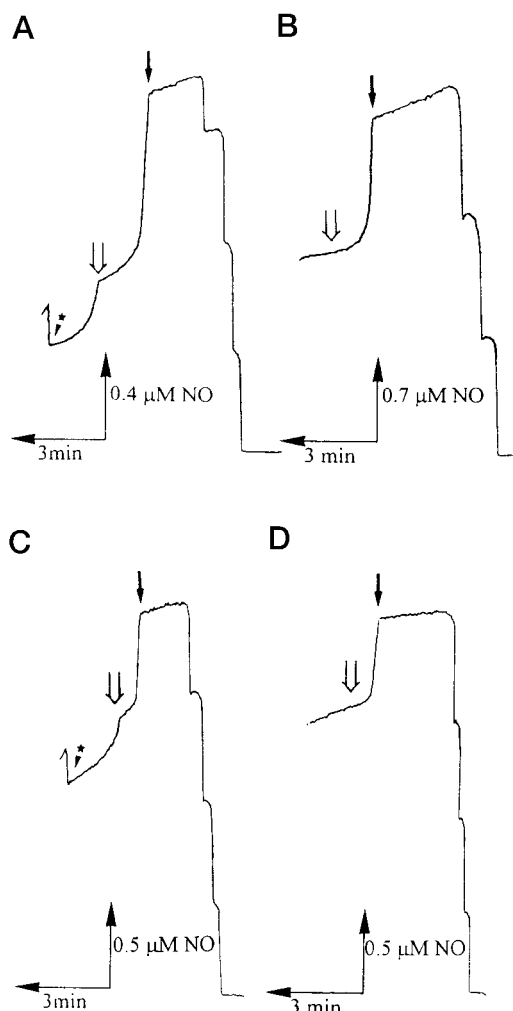
FIG. 3. **S-Nitrosation of deoxy- and oxy-Hb.** *Top traces* (*A* and *B*), deoxy-Hb (0.2 μM tetramer; *A*) or Hg^{2+}-treated deoxy-Hb (0.325 μM tetramer; *B*) were anaerobically added ($\downarrow$) to the electrode reaction chamber containing 1.8 and 3.0 μM NO, respectively. The subsequent fast disappearance of NO is due to NO binding to the hemes of Hb in an essentially 1:1 stoichiometry. Thereafter in sequence, 10 μM Cu^{2+} was added ($\Downarrow$) and in *A* ~0.2 μM NO disappeared due to *S*-nitrosation of Hb; an almost equivalent amount of NO was released upon addition of 20 μM Cu$^+$ ($\star$). Notice that in *B* the addition of Cu^{2+} ($\Downarrow$) did not cause further changes in the NO concentration, indicating that treatment with Hg^{2+} prevents the *S*-nitrosation reaction. *Bottom traces* (*C* and *D*), oxy-Hb (*C*) or oxy-Hb treated with PMB (*D*) (0.125 μM tetramer) were anaerobically added ($\downarrow$) to the electrode reaction chamber containing 2.0 μM NO. Following addition of 10 μM Cu^{2+} ($\Downarrow$), in *C* ~0.25 μM NO further disappeared and was then released upon addition of 20 μM Cu$^+$ ($\star$); the same addition of Cu^{2+} in *D* was ineffective, confirming that treatment with PMB prevents the *S*-nitrosation reaction of oxy-Hb. Number of replicate experiments, $n = 5$.

The same experiment carried out in the presence of 70 μM neocuproine showed a similar profile, but a remarkable increase of the reaction efficiency (Fig. 6, *data set b*); the complete disappearance of free cysteines is already observed at a Cu^{2+}-to-BSA ratio of 4:1. Taken together, these findings imply that formation and stability of BSA-SNO crucially depend on the relative concentrations of NO, Cu^{2+}, BSA, and Cu$^+$ (the latter likely formed in the reaction between BSA and Cu^{2+}).

DISCUSSION

We found that, in the presence of NO, cupric ions can induce a fast *S*-nitrosation of both BSA and Hb, used as model systems, whereas ferric ions are in this respect ineffective. This conclusion is based on amperometric and spectroscopic measurements. (i) Amperometric experiments (Fig. 2) showed that
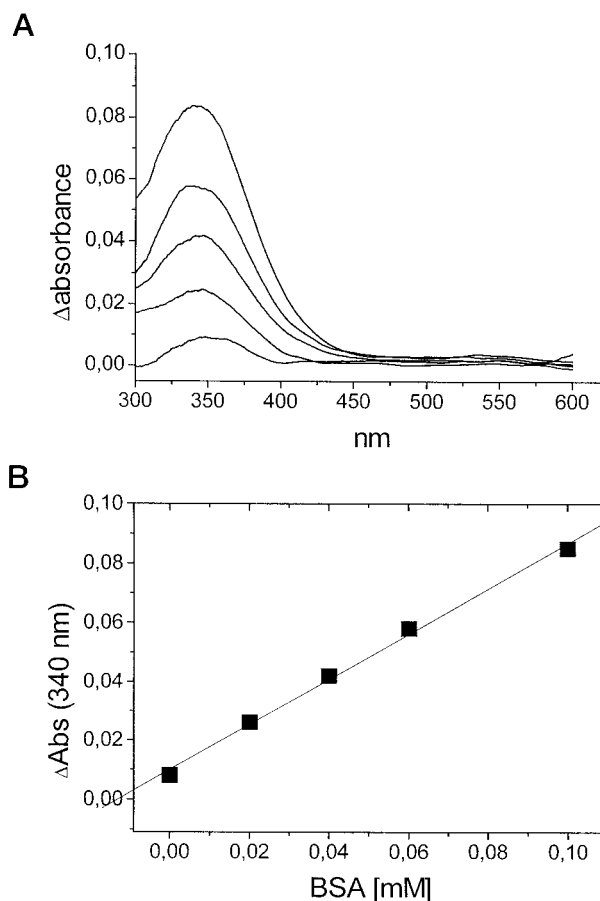

FIG. 4. **Spectral changes of BSA-SNO formation.** *A*, spectra collected between 300 and 600 nm after a 3-min incubation of 0, 20, 40, 60, and 100 μM BSA in air-equilibrated 0.1 M Hepes buffer, pH 7.5, with 200 μM NO and 0.5 mM Cu^{2+}. To subtract the optical contribution of copper ions, parallel experiments were performed in the absence of NO. The absorbance change observed in the 340 nm region reflects the formation of BSA-SNO; the residual absorbance observed in the absence of BSA is likely due to nitrite formed in the reaction of NO with oxygen and is responsible for the intercept of the curve in *panel B*. *B*, linear regression analysis of the absorbance change at 340 nm, yielding $\epsilon = 0.77$ mM^{-1} cm^{-1} for BSA-SNO.
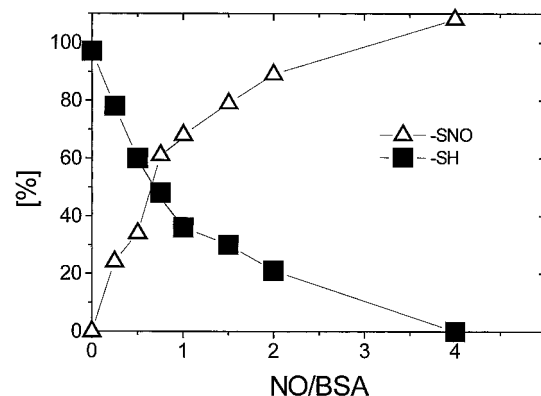

FIG. 5. **Effect of NO on BSA-SNO formation.** 50 μM BSA was incubated aerobically for 3 min with 200 μM Cu^{2+} and 0–200 μM NO. Upon the addition of 1 mM EDTA to remove excess copper, BSA-SNO was calculated from the absorbance at 340 nm and thiol concentration from the absorbance at 412 nm after addition of 100 μM DTNB.

NO disappears from solution in a ~1:1 NO-to-BSA ratio in the presence of both BSA and Cu^{2+}, but not in the presence of either BSA or Cu^{2+} alone. BSA-SNO formation is indicated by the NO release observed upon addition of Cu$^+$ to the reaction mixture, which is known to cause the decomposition of RS-NOs
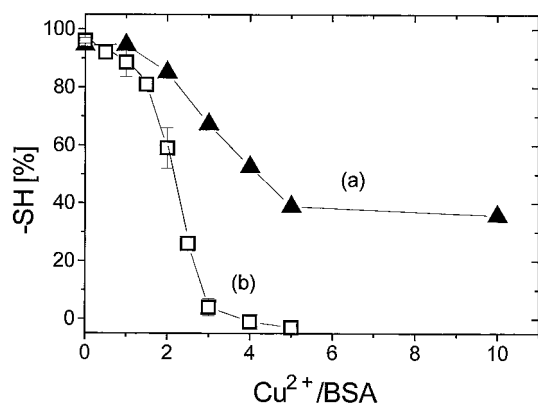
FIG. 6. **Effect of Cu^{2+} on BSA thiol concentration.** 7 μM BSA was incubated aerobically for ~3 min with 35 μM NO and increasing amounts of Cu^{2+} in the absence (*a*) and presence (*b*) of 70 μM neocuproine. Upon addition of 1 mM EDTA, to remove excess copper, 100 μM DTNB was added and the thiol concentration was calculated from the absorbance at 412 nm.

SCHEME 1

SCHEME 2

with a release of NO (12, 18). Similar experiments carried out with Hb induced its *S*-nitrosation with an observed stoichiometry dependent on the Hb derivative used, *i.e.* oxygenated or deoxygenated (Fig. 3). (ii) Spectroscopic measurements showed that the reactive Cys-34 of BSA decreased close to zero if the protein was incubated with NO and Cu^{2+}, but not with either one of the two reagents separately (Fig. 1). (iii) Formation of BSA-SNO was followed by the characteristic absorbance of the *S*-NO bond (maximum at 340 nm) in samples where increasing concentrations of BSA were incubated with NO and Cu^{2+} (Fig. 4); the extinction coefficient at 340 nm was determined, $\epsilon = 0.77$ mM^{-1} cm^{-1}, in agreement with the literature (35). (iv) Upon addition of increasing amounts of NO to BSA in the presence of Cu^{2+}, we observed a decrease of the free thiol concentration and a parallel increase of the amount of BSA-SNO (Fig. 5). The sum of free thiol and *S*-NO accounted for the total amount of thiols independently calculated.
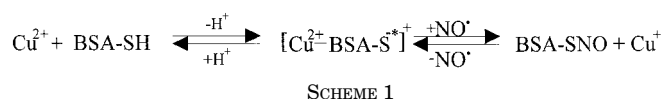
The yield of BSA-SNO in experiments carried out in the absence or presence of O_2 was the same. Based also on the finding that no reaction was observed following the aerobic incubation of BSA with NO (in the absence of Cu^{2+}), we can exclude a role of oxygen in this process (see Fig. 2A). The concentration of BSA was, in any case, much too low (micromolar) to efficiently compete with water in the reaction with N_2O_3 formed in the auto-oxidation reaction of NO (21).

Taken together, the evidence summarized above suggests the mechanism shown in Scheme 1 for the copper-mediated formation/degradation of BSA-SNO.

The sulfhydryl group of Cys-34 of BSA binds Cu^{2+} forming a copper-thiol complex, which reacts with NO to yield BSA-SNO. This mechanism is proposed as both Cu^{2+} and BSA are needed for the reaction with NO; it is interesting to notice that Fe^{3+} was in this respect ineffective. The formation of a copper-BSA complex is also suggested by the experiments performed in the presence of excess neocuproine; the formation of a Cu^+-neocuproine complex, characterized by a weak absorbance between 400 and 500 nm (36), was in fact observed only upon addition of NO to BSA and Cu^{2+}, indicating that (i) the copper-BSA complex is itself rather stable (minutes) and (ii) the release of Cu^+ may occur only after the attack of NO to form an *S*-NO bond.

The mechanism presented in Scheme 1 is novel, insofar as the copper-mediated reaction of NO with thiols does not directly involve NO$^+$, as previously suggested (8, 21, 23, 24) and here reported (Scheme 2).

NO$^+$ is known to react with water to form nitrite; therefore, in the presence of Cu^{2+} the equilibrium depicted in Scheme 2
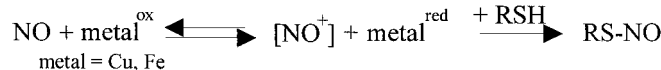
should be shifted toward NO$^+$ and NO should rapidly disappear. However, this was not observed in the amperometric measurements reported in Fig. 2, which demonstrate instead that, even in the presence of a 5-fold excess of Cu^{2+}, no significant disappearance of free NO occurs, a finding that rules out a significant role of NO$^+$ in RS-NO formation at least at micromolar amounts of Cu^{2+}. Scheme 1 seems also to be supported by the standard reduction potentials, which favor the reduction of copper ions by thiols rather than by NO (see Table I). Indeed, it is known that Cu^{2+} readily oxidizes GSH and cysteine (37).

The kinetics of formation, as well as the structure of copper-thiol complexes, particularly those with GSH or cysteine, are well described in the literature: Cu^{2+} is chelated by the thiolate sulfur of glutathione probably forming a 2:1 Cu^{2+}-GSSG complex (38, 39); both the complex-formation and the involved redox reaction (see Scheme 3, taken from Ref. 38) are estimated to be very fast (~10^8 M^{-1} s^{-1} and ~110 s^{-1}; Ref. 18).

A more general mechanism, including the reactions depicted in Schemes 1 and 3 and the Cu^+-induced decomposition of RS-NO, is reported in Scheme 4.

This model provides also a simple explanation for the totally different reactivities of the "small" glutathione and the "large" BSA or Hb with Cu^{2+} and NO. The addition of GSH to the electrode chamber containing NO and Cu^{2+} was not followed by disappearance of NO, while the incubation of GSH with Cu^{2+} in a spectroscopic cuvette led to complete disappearance of thiols (Fig. 1B). Both findings can be explained assuming that Cu^{2+} induces prompt dimerization of GSH, the reaction being in kinetic competition with the formation of GSNO. Judging from our results, although the reaction of NO with glutathione may be fast, it is overruled by the faster dimerization (see Scheme 4). This is not surprising as the activated thiols in the Cu^{2+}-glutathione complex are in very close proximity (~5 Å, taken from crystallographic data; Ref. 38).

In contrast, the lifetime of the BSA-copper complex is longer since dimerization and disulfide formation is hindered by steric restriction. Radi *et al.* (40) reported that dimerization of BSA is only observed under denaturing conditions, and oxidation to sulfenic or sulfinic acid only occurs in the presence of strong oxidants, such as peroxynitrite. Consistently with these observations, following incubation of BSA with Cu^{2+} (15 min), we did not detect a decrease of the free thiol concentration. This finding excludes fast oxidation of Cys-34 and dimerization of BSA, allowing study of the role of Cu^{2+} in the reaction of thiols with NO.

The results shown in Fig. 6 indicate that Cu^{2+} enhances in a concentration-dependent manner the *S*-nitrosation of BSA. The formation of BSA-SNO strictly depends (at constant NO) on the relative concentrations of Cu^{2+} and BSA, although up to a Cu^{2+}/BSA ratio of 1:1 no disappearance of thiols was observed. The further increase of Cu^{2+} led to a sharp decrease of free thiols. This finding is consistent with BSA having two copper binding sites, as also suggested by Kashiba-Iwatsuki *et al.* (31); the first (high affinity) site is inactive, and the second is involved in the reaction with NO. Kashiba-Iwatsuki *et al.* (31) suggested that Cu^{2+} binds to BSA with high affinity to the

TABLE I
Standard reduction potentials taken from Refs. 32 and 44

$Cu^{2+} + e^-$ ⇌ Cu^+		0.15 V
$1/2\ GSSG + H^+ + e^-$ ⇌ GSH		-0.30 V
$NO^+ + e^-$ ⇌ NO		1.2 V
$Fe^{3+} + e^-$ ⇌ Fe^{2+}		0.77 V

SCHEME 3

SCHEME 4
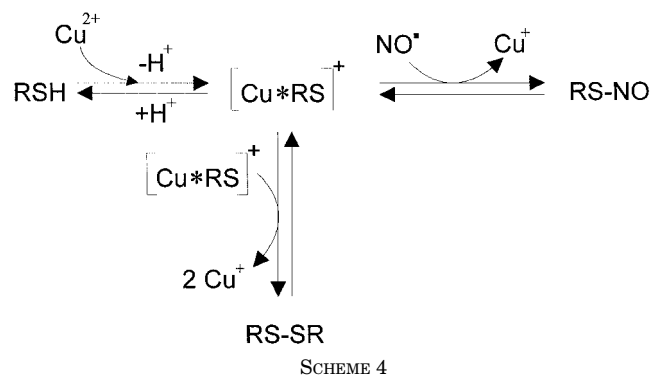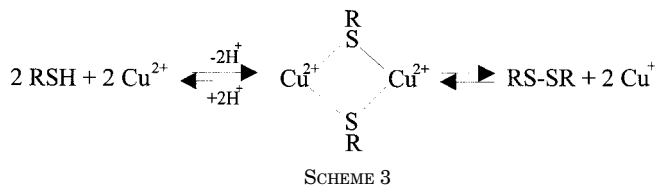
N-terminal domain (amino group of Asp-1 and imidazole group of His-3) and with lower affinity to Cys-34. Therefore, Cu^{2+} would first saturate the high affinity binding site and then react with Cys-34 allowing BSA-SNO formation, in the presence of NO. Blocking the thiol with Hg^{2+} or NEM completely prevented the reaction with NO.

The incomplete formation of BSA-SNO observed amperometrically and spectroscopically is probably related to the presence of Cu^+ formed in the reaction of Cu^{2+} with BSA (see Schemes 1 and 4). As reported above, Cu^+ is known to catalyze the decomposition of RS-NOs releasing NO and disulfides (12, 18). *In situ* formation of Cu^+ would therefore decompose BSA-SNO favoring the back reaction. The extent of the RS-NO formation, should therefore depend on the Cu^+/Cu^{2+} ratio, controlling the relative formation and decomposition kinetics. As expected, experiments performed in the presence of neocuproine, a specific Cu^+ chelator (with lower affinity for Cu^{2+}), remarkably increased the efficiency of BSA-SNO formation (Fig. 6).

As proved by the prompt Cu^{2+}-mediated nitrosation of Hb, followed by the release of NO induced by Cu^+, the mechanism presented can also be viewed as a new synthetic method for *in vitro* S-nitrosation of proteins. It displays several advantages over conventional methods. Commonly, S-nitrosation is achieved by incubation with low molecular weight RS-NOs like GSNO or S-nitrosocysteine. To allow complete reaction they have to be used in large excess over the protein and therefore, to check the yield of protein nitrosation, often a separation step is necessary. As the reactions described here are efficient, complete formation of RS-NO is achieved even at micromolar concentrations of reactants; under these conditions the reaction can be directly followed either spectroscopically or amperometrically, without the need of a separation step.

Finally, it may be of interest to speculate also on a role of copper ions in the intra- and extracellular NO traffic. However, most of Cu^{2+} is incorporated into ceruloplasmin (95% of plasma copper; Ref. 41), metallothioneines, and albumin (41); therefore, the extra- and intracellular concentration of "free" copper ions is very low (42, 43). Although we cannot assess a physiological relevance of the reactions presented, it would be appropriate to further investigate a possible role for copper ions in *in vivo* S-nitrosation.

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Mechanism of *S*-Nitrosothiol Formation and Degradation Mediated by Copper Ions

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