Sodium Nitroprusside: Mechanism of NO Release Mediated by Sulfhydryl-Containing Molecules

Loris Grossi* and Sandra D'Angelo

Dipartimento di Chimica Organica "A. Mangini", Università di Bologna, Viale Risorgimento 4, I-40136 Bologna, Italy

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Sodium nitroprusside (SNP) is among the most widely studied nitric oxide donors, and its capability of producing NO seems to depend on its interaction with sulfhydryl-containing molecules present in vivo. The aim of this research has been the study of the mechanism of interaction between SNP and sulfhydryl-containing compounds, such as cysteine and glutathione, through detection by EPR, UV−vis, and IR spectroscopy of both the radical and nonradical species involved. An electron-transfer process can be invoked as the key step, which leads to the formation of the reduced SNP radical, the main detectable radical intermediate, and the corresponding *S*-nitrosothiol, the ending product of NO that can be considered the real storage and transporters of NO. When cysteine was used, a second radical species (**A**) is detectable: it can be accounted for by the interaction of a byproduct with unreacted cysteine.

Introduction

The vasodilator effects of sodium nitroprusside (sodium pentacyanonitrosylferrate, SNP) are believed largely mediated by NO. In vivo, the mechanism of release of this molecule from SNP is hypothesized to involve the sulfhydryl-containing compounds glutathione and cysteine, leading to the formation of the corresponding disulfides and *S*-nitrosothiols, NO, and cyanide ions. Actually, the reaction between SNP and thiols was described several decades ago[1] and is used as a test for their identification, but still, although SNP is the most widely studied of the iron nitrosyl compounds, the mechanism of NO release is far from completely elucidated. In particular, it has been suggested that radical species are involved, but ambiguous results are reported in the literature.[2−6] To verify this hypothesis, and clarify the reaction mechanism, experiments in distilled water, pH = 7, and phosphate buffer solutions, pH = 7.4, 6.86, 6.4, and 5.0, have been carried out and investigated by EPR, IR, and UV−vis spectroscopy.

Results and Discussion

Sodium nitroprusside, $Na_2[Fe^{III}(CN)_5(NO)]$, is a nonferromagnetic species,[7] which easily in solution can be reduced to the paramagnetic $[Fe^{II}(CN)_5(NO)]^{3-}$. Thiols, such as cysteine (CySH) and glutathione (GSH), are well-known to perform this process.[5]

To confirm this behavior, EPR experiments on the interaction between SNP and these biological thiols were conducted. The same radical species, characterized by a three-line EPR spectrum, whose spectroscopic parameters (a_N = 1.48 mT, *g*-factor = 2.0255) are in agreement with those reported[2,3] for the reduced SNP radical, was immediately detected with the glutathione (Figure 1), as well as the cysteine (Figure 3a).

In the literature,[2,3] for the latter radical a spontaneous decay via *trans*-cyanide ligand dissociation was invoked, and the resulting $[Fe(CN)_4NO]^{2-}$ was the only ultimate detectable radical of the first reduction process; then, via a further reduction, a second radical species, considered the main ending species, should become detectable, even if never clearly identified.[8−10]

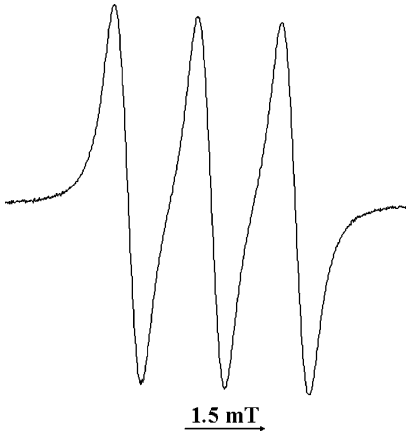

Figure 1. The reduced SNP radical detected in the reaction between SNP and GSH, after 1 min.

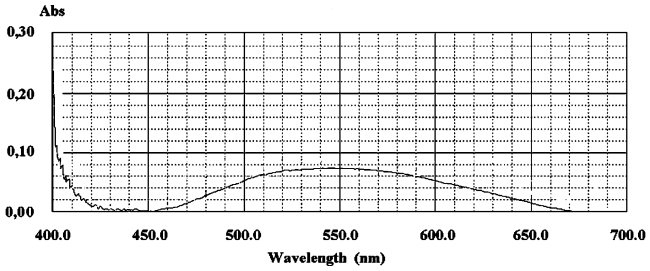

Figure 2. UV−vis spectrum recorded in an experiment conducted in mixing-flow mode with two buffer solutions, pH 7.4, of SNP and GSH, respectively; both solutions were 0.08 M.

These data let us suppose that if other radical species from the following reactions[5,6,10,11] were formed, new EPR signals should come out at the expense of that of the reduced SNP. Thus, an experiment with SNP and GSH was directly conducted in the cavity of an EPR

* Corresponding author. Phone: +39-051-2093627. Fax: +39-051-2093654. E-mail: grossi@ms.fci.unibo.it.

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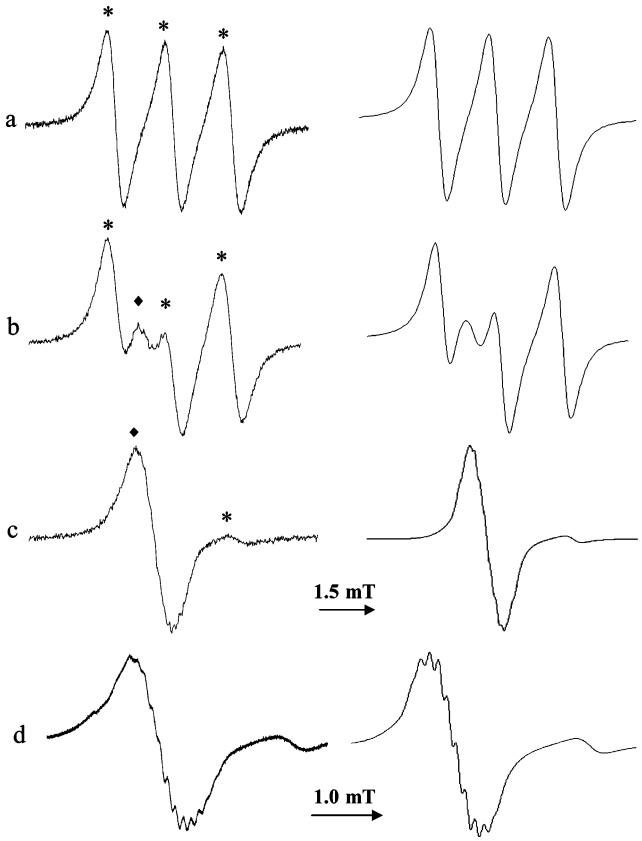


Figure 3. Radical species detected in the reaction between SNP and CySH under the same experimental condition as for GSH. (*)reduced SNP radical, (◆) radical **A**: (a) spectrum detected after 1 min, (b) after 5 min, and (c) after 25 min and (d) spectrum, after 25 min, in expanded scale and higher resolution. Right column: corresponding computer-simulated spectra.

spectrometry, and the advance of the reaction, at room temperature, was continuously monitored over an extended period of time: no evidence of the formation of other radical species,[9] except the reduced SNP (Figure 1) was achieved.

The strong discrepancy between our results and those reported in the literature[5−6,10−11] required us to prove unambiguously the structure of the radical we detected. For this purpose, different reducing agents, such as sodium borohydride, $NaBH_4$, and sodium naphthalenide, were tested. Only one radical species, characterized by the same EPR spectroscopic parameters of that obtained when the GSH or the CySH were reacting, was detectable (data not shown). This result supported our findings, i.e., that the reduced SNP, $[Fe(CN)_5NO]^{3-}$, is the primary detectable radical species in the reaction between SNP and GSH or CySH and that no iron−nitrosyl−sulfur complexes, in general described as Fe−NOSR,[4,10,18] are detectable. However, stronger evidence was needed to support these results.

In principle, the study of the reaction 1, a redox equilibrium that is reported to be shiftable back via

$$[Fe(CN)_5\,NO]^{2-} \underset{\text{Oxidant}}{\overset{\text{Reducing}}{\rightleftharpoons}} [Fe(CN)_5\,NO]^{3-} \qquad (1)$$

SNP SNP-reduced

aerobic oxidation,[2,4,12−13] could prove or exclude the formation of radicals due to following processes. Thus, the reaction between a carefully deoxygenated DMF solution of SNP and sodium naphthalenide, added step by step, under anaerobic conditions, was directly conducted in an IR spectrometer cell and continuously monitored. Besides the presence of the SNP, the reduced SNP was detectable too, whose concentration was increasing at the expenses of SNP upon addition of the reactant. When the reducing agent supplemented was equimolar to the starting nitroprusside, the SNP vanished and only the absorption bands due to the reduced SNP remained evident. At this stage, air, the oxidant, was allowed inside the sample cell: immediately the IR spectrum showed the peak from SNP rising and concomitantly that of reduced SNP diminishing (data not shown). This result proved that a direct electron transfer was taking place between the reactants (reaction 1) and no involvement of possible intermediates, for example adducts of the reducing agent to the SNP, usually considered a precursor of the reduced SNP,[4−5,10] seemed achievable. Furthermore, this experiment evidenced that the formation of other radical species, due to a spontaneous dissociation of the reduced SNP, if it takes place, as sometimes invoked, is very low.

However, although EPR and IR evidence stressed the persistence of the reduced SNP radical, the NO release has to take place. The decay of reduced SNP via cyano trans-elimination, the poisoning effect in vivo,[9,14] followed by NO release is well-known, but in the light of the mentioned results, it would seem to be slow and therefore incapable to account for the efficiency of SNP as NO supplier in urgent therapy.[15] Because of the claimed fundamental role played by the sulfhydryl-containing compounds, if GSH was involved, a glutathione radical cation ($GSH^{\bullet+}$), leading to the formation of GSNO,[16] could be hypothesized (Scheme 1).

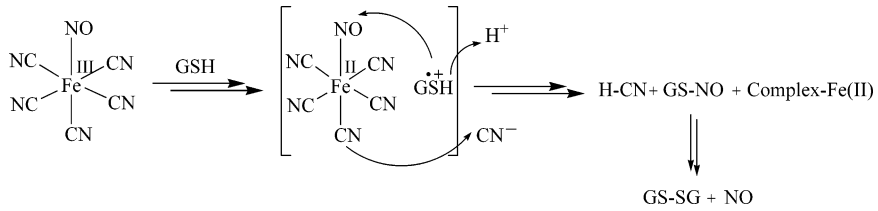
This mechanism would account for not only the trans-elimination of the cyanide group, via the formation of the protonated cyanide,[17] but also for the crucial role played by the thiol group, which acts as reducing agent, forming GSNO[16] as storage and carrier of NO. To verify this hypothesis, i.e., the formation of GSNO, the reaction between SNP and GSH was conducted directly within the UV−vis spectrometer cell; the absorption spectrum in the 530−560 nm region[18] let us identify the formation of GSNO (Figure 2), which could be considered strong support for the mechanism we proposed.

Actually, this result was in accord with those reported in an analytical quantitative study[15] about the detection (ITP method) of the corresponding *S*-nitroso derivatives formed in the reaction between the SNP and thiols such as *N*-acetyl-L-cysteine, GSH, and *N*-acetyl-D,L-penicillamine.

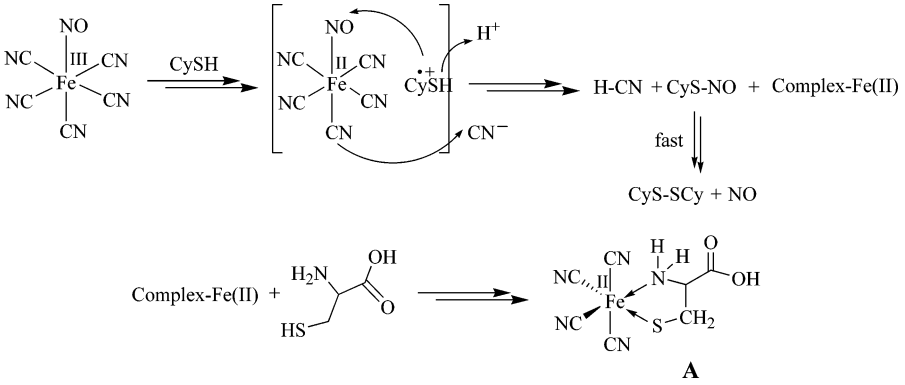
Even if the reaction between SNP and cysteine had been deeply investigated,[5,11−12] it was necessary to prove our hypothesis also with this amino acid. As stated before, the reaction between SNP and cysteine let us detect immediately the reduced SNP radical (Figure 3a), but upon monitoring the advance of the reaction, a new radical species shortly grew at expenses of the former (Figure 3b,c). This new species, characterized by a single-line EPR spectrum, *g*-factor = 2.0297, was totally absent in experiments with GSH.

The discrepancy between results obtained with glutathione and cysteine required further investigation of

Scheme 1. Reaction Mechanism Proposed



Scheme 2. Reaction Mechanism Accounting for the Formation of Radical A Detected in Experiments with Cysteine



the role played by the sulfhydryl compound. Thus, different thiols, such as benzyl thiol and 3-mercaptopropionic acid, were studied; both thiols, when reacted with SNP under the same conditions as for GSH and CySH, led to detection of the reduced SNP radical only.

In the light of these results, the unforeseen radical species, which goes with the cysteine, could be inferred, following the literature,[2] to $[Fe(CN)_4NO]^{2-}$, or a species due to a subsequent reduction of it, or an iron−nitrosyl−sulfur complex.[19] To account for the EPR hyperfine structure (Figure 3d), a radical species with four H-atoms and one N-atom in its structure has to be hypothesized, as supported by computer simulations, but none of the radicals hypothesized in the literature could match it. Actually, a similar EPR pattern was reported[19−21] for species obtained in the reaction of Fe(II) ($FeSO_4$) with gaseous NO and cysteine, and it was ascribed to a paramagnetic dinitrosyl−iron complex $(NO)_2Fe^{II}[SCH_2CH(NH_2)COOH]_2$ characterized by hyperfine coupling constants due to two equivalent nitrogen atoms ($a_N = 0.235$ mT), the NO groups, and four equivalent hydrogen atoms ($a_H = 0.121$ mT) belonging to the two methylene groups ($S-CH_2$). However, it seems really difficult to hypothesize an analogous structure for the radical species we detect. In fact, to form such a radical species, the release of all five cyano groups should take place,[10] to get free Fe(II), and even if we thought this was feasible, we must assume the presence in the medium of a large amount of free NO. Actually, the only source of NO is the reaction between SNP and the reducing species, which in principle should lead to a NO derivative able to store and transport the nitric oxide. Finally, the coordination of SR groups to the iron should be totally independent of the sulfhydryl compound used; i.e., we should detect this type of radical in all experiments.

To account for our result, it seemed reasonable to hypothesize that the iron complex, resulting from the release of both the cyanide ion and the NO, could interact with another molecule of CySH and lead to a new paramagnetic hexacoordinate complex (**A**) (Scheme 2).

Actually, the radical species **A** can account for the EPR spectrum we detect, i.e., three hydrogen atoms with coincident hyperfine coupling constant ($a_H = 0.12$ mT), most probably one hydrogen belonging to the $-NH_2$ group and two to the methylene of the $-SCH_2$ group, and one nitrogen and one hydrogen atom, with coincident hyperfine coupling constant ($a_N = 0.245$ mT), both belonging to the amino group (Figure 3d). To prove the hypothesized structure, attempts were then carried out using deuterated cysteine, in particular the $-ND_2$ derivative.[22] In fact, if the structure of radical **A** is that which we proposed, a change in the hyperfine structure should be evidenced in the EPR spectrum, thus allowing clear identification of the atoms involved. Unfortunately, the hyperfine structure could not be resolved, also in an expanded scale. Most probably, this time, the EPR spectrum shows two contemporary radical species,[22] i.e., the cysteine and the cysteine$-ND_2$ adducts. However, indirectly, this result confirmed our hypotesized structure. In fact, if a dinitrosyl−iron complex was the radical species detected, its spectrum should be totally unaffected by the presence of the $-ND_2$ group; i.e., the same hyperfine structure should still be evidenced. Furthermore, the structure of **A** could also validate the behavior of sulfhydryl compounds, such as benzyl thiol and 3-mercaptopropionic acid, which do not allow detection of an analogous radical: in these substrates the amino group is absent. On the contrary, when GSH is used, the nondetection of this type of radical, which in principle should be formed, it is most probably due to the bulky structure of this molecule; actually, simple modeling calculations support that cysteine alone can assume the right conformation for a possible coordination with the iron complex. At this stage, we needed to prove if both requirements, i.e., the presence of a nitrogen atom and a sulfur atom, in γ-position each other, and the right geometry, are compulsory for

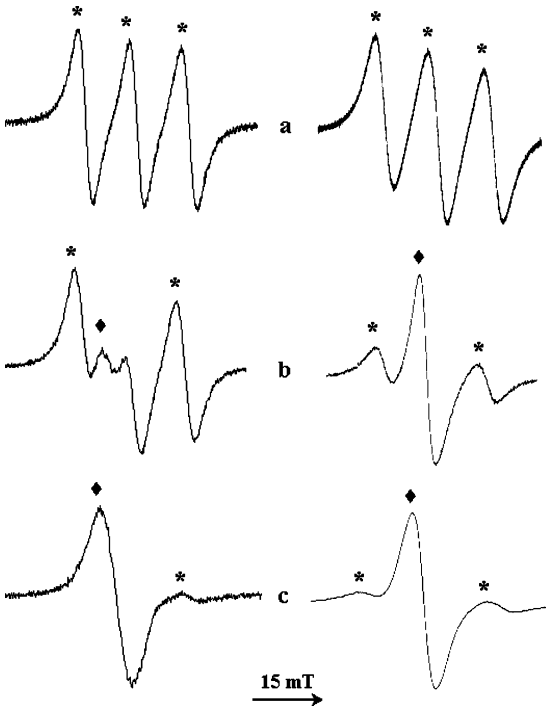


Figure 4. Left column: Radical species detected in the reaction between SNP and CySH; see Figure 3a−c. Right column: Radical species detected in the reaction between SNP and 2-aminothiophenol recorded under the same experimental condition as for CySH. (*) reduced SNP, (◆) radical type **A**, *g*-factor 2.0027. (a) spectrum detected after 1 min,; (b) after 5 min, (c) after 25 min.

generating a radical species such as **A**. These conditions could be fulfilled by the 2-aminothiophenol. EPR experiments were then conducted with this reactant under the same conditions as for the cysteine: the spectrum showed the same trend (Figure 4, right column).

All these results strengthen our hypothesis that considers the reduced SNP as the prevailing radical species formed in the reaction between SNP and sulfhydryl-containing compounds, detectable by EPR. Other radical species, sometimes evident, have to be considered as deriving from byproducts of the main process.

Finally, it was necessary to prove that in the key step of the interaction between the sulfhydril derivatives and SNP, i.e., the electron-transfer mechanism, the reducing agent involved is not necessarily the thiolate, as often reported in the literature.[2a,5,11−12] Thus, experiments between SNP and GSH were conducted in three different acidic buffer solutions, pH = 6.86, 6.4, and 5.0, in which the percentage of thiolate is estimated to be roughly 0.85, 0.30, and 0.01, respectively. In all the experiments, the reduced SNP was straightforwardly detectable by EPR. That definitely supported the possibility of the involvement of the thiol group itself as reducing species.

Conclusions

Even if for the mechanism of NO release from the nitroprusside, in vivo, different routes could be involved, our results definitely seem to support the fundamental role played by sulfhydryl-containing compounds, and in particular, the glutathione and the cysteine could have a crucial role. The intermediacy of the radical species confirms that the NO release takes place through an electron-transfer mechanism and not via a direct iron−nitrosyl bond cleavage, as invoked several time. In contrast, with most of the literature[5,11−12] we think that the thiol group, and not necessarily its corresponding thiolate, is directly involved in the electron-transfer process, as proved by EPR results obtained in experiments reacting SNP and glutathione in acidic buffer solutions, all allowing detection of the reduced SNP radical. The formation of *S*-nitrosothiols, which are considered the storage and the transporters of NO in vivo, allowed the hypothesis that the SNP action in hypertensive emergencies,[15] i.e., its rapid releases of NO, could be due to the spontaneous release[23,24] of NO that these ending products can perform. Finally, a second radical species, **A**, is detectable just in the experiments with CySH; it can be accounted for via interaction of CySH with a byproduct derived from the decay of the reduced SNP radical.

Experimental Section

All experiments were performed at room temperature. EPR experiments were conducted in water, pH = 7, and in aqueous buffered solutions at pH = 7.4, 6.86, 6.4, and 5.0, using a "H-shaped" quartz sample-tube to keep initially separate the two reactants (both 0.1 mmol). The thiol aqueous solution was introduced in one of the two branches, while the SNP, as a solid, was introduced in the other. The solution was degassed by means of the freeze−pump−thaw technique and then the tube sealed off. The two reactants were mixed and the solution immediately analyzed, at room temperature, by the EPR spectrometer. All spectra have been recorded using the following instrumental parameters: microwave frequency, 9.75 GHz; modulation frequency, 100 kHz; microwave power, 10 mW; receiver gain, 2.0×10^4; modulation amplitude, 0.96 G; and time constant, 2.56 ms. The spectrum showed in Figure 1d used the following parameters: receiver gain, 4.0×10^4; microwave power, 6 mW; modulation amplitude, 0.23 G; and time constant, 5.12 ms. For experiments with sodium naphthalenide (0.1 mmol) and $NaBH_4$ (0.1 mmol), high-grade dry THF and CH_3OH, respectively, were used as solvent. The cysteine-ND_2 was obtained by dissolving cysteine in pure CD_3OD.

IR experiments were conducted under both anaerobic and aerobic conditions; the reacting mixture, SNP and sodium naphthalenide, was prepared in DMF (dimethylformamide) as solvent, at a final concentration 10^{-4} M.

UV−vis experiments were conducted using an appropriate flow-cell. Two gastight syringes containing carefully deoxygenated buffer aqueous solutions of GSH and SNP, respectively, both 0.08 M, were flowed by a syringe−pump apparatus in a mixing chamber placed just immediately before the spectrometer flow-cell. The formation of GSNO (Abs = 0.075, ca. 1.1×10^{-2} M) was monitored continuously (Figure 2). The GSNO molar extinction coefficient, $\epsilon_{546} = 6.60 \pm 0.01$ mol^{-1} dm^3 cm^{-1}, was determined by direct measurement of a 0.01 M solution of pure GSNO in buffered solution at pH = 7.4. The formation of CySNO too was spectroscopically evidenced, but its fast decay did not allow a quantitative measurement.

The product analysis, when glutathione and cysteine were reacted, allowed identification, by H NMR spectroscopy, of cystine and oxidized glutathione, respectively.

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References

(1) (a) Scagliarini, G. *Atti V Congr. Naz. Chim. Pure Appl.*, Rome, 1935. (b) Swinehart, J. H. The nitroprusside ion. *Coord. Chem. Rev.* **1967**, *2*, 385−402.

(2) (a) Butler, A. R.; Calsy-Harrison, A. M.; Glidwell, C.; Sorensen, P. E. The pentacyanonitrosylferrate ion-V. The course of the reactions of nitroprusside with a range of thiols. *Polyhedron* **1988**, *7*, 1197−1202. (b) Butler, A. R.; Calsy, A. M.; Johnson, I. L. Enzyme inhibition by sodium nitroprusside. *Polyhedron* **1990**, *9*, 913−919. (c) Butler, A. R.; Megson, I. L. Non-Heme Iron Nitrosyls in Biology *Chem. Rev.* **2002**, *102*, 1155−1165.
(3) Leeuwenkamp, O. R. Ph.D. Thesis, University of Leiden, 1985.
(4) Rochelle, L. G.; Kruszyna, H.; Kruszyna, R.; Barchowsky, A.; Wilcox, D. E.; Smith, R. P. Bioactivation of Nitroprusside by Porcine Endothelial Cells. *Toxicol. Appl. Pharmacol.* **1994**, *128*, 123−128.
(5) Szacilowski, K.; Wanat, A.; Barbieri, A.; Walsielewska, E.; Witko, M.; Stochel, G.; Stasicka, Z. Reactions of the $[Fe(CN)_5NO]^{2-}$ complex with biologically relevant thiols. *New J. Chem.* **2002**, *26*, 1495−1502.
(6) (a) Schwane, J. D.; Ashby, M. T. FTIR Investigation of the Intermediates Formed in the Reaction of Nitroprusside and Thiolates *J. Am. Chem. Soc.* **2002**, *124*, 6822−6823. (b) Carapuca, H. M.; Simao, J. E. J.; Fogg, A. G. Comproportionation and disproportionation reactions in the electrochemical reduction of nitroprusside at a hanging mercury drop electrode in acidic solution. *J. Electroanal. Chem.* **1998**, *455*, 93−105.
(7) A different description of SNP, formally containing Fe(II) and NO^+, is reported; see: Manoharan, P. T.; Gray, H. B. Electronic Structure of Nitroprusside Ion. *J. Am. Chem. Soc.* **1965**, *87*, 3340−3348.
(8) Glidewell, C.; Johnson, I. L. Definitive identification of the primary reduction product of the nitroprusside ion, pentacyanonitrosylferrate(2−), in aqueous solution *Inorg. Chim. Acta* **1987**, *132*, 145−147.
(9) Cheney, R. P.; Simic, M. G.; Hoffman, M. Z.; Taub, I. A.; Asmus, K. D. One-electron reduction of pentacyanonitrosylferrate(II) ion in aqueous solution. *Inorg. Chem*. **1977**, *16*, 2187−2192.
(10) Wilcox, D. E.; Kruszyna, H.; Kruszyna, R.; Smith, R. P. Effect of Cyanide on the Reaction of Nitroprusside with Hemoglobin: Relevance to Cyanide Interference with the Biological Activity of Nitroprusside. *Chem. Res. Toxicol.* **1990**, *3*, 71−76.
(11) Johnson, M. D.; Wilkins, R. G. Kinetics of the Primary Interaction of Pentacyano-nitrosylferrate(2−) (Nitroprusside) with Aliphatic Thiols. *Inorg. Chem.* **1984**, *23*, 231−235.
(12) Morando, P. J.; Borghi, E. B.; de Schteingart, L. M.; Blesa, M. A. The Reaction of Cysteine with Pentacyanonitrosylferrate(2−) Ion. *J. C. S. Dalton* **1981**, 435−440.
(13) Roncaroli, F.; Olabe, J. O.; van Eldik, R. Kinetics and Mechanism of the Interaction of Nitric oxide with Pentacyanoferrate(II). Formation and Dissociation of $[Fe(CN)_5NO]^{3-}$. *Inorg. Chem.* **2003**, *42*, 4179−4189.
(14) Rindone, J. P.; Sloane, E. P. Cyanide toxicity from sodium nitroprusside: Risks and management *Ann. Pharmacother.* **1992**, *26*, 515−519.
(15) Feelisch, M. The use of nitric oxide donors in pharmacological studies. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *358*, 113−122.
(16) Tsikas, D.; Böger, R. H.; Bode-Böger, S. M.; Brunner, G.; Frölich, J. C. Formation od S-nitroso compounds from sodium nitroprusside, nitric oxide or nitrite and reduced thiols: Analysis by capillary isotachophoresis. *J. Chromatogr.* **1995**, *A, 699*, 363−369.
(17) It needs to be pointed out that in an aqueous medium the acid−base dissociation constant for HCN is $K_a = 5.85 \times 10^{-10}$.
(18) Goldstein, S.; Czapski, G. Mechanism of the Nitrosation of Thiols and Amines by Oxygenated NO Solutions: The Nature of the Nitrosating Intermediates. *J. Am. Chem. Soc*. **1996**, *118*, 3419−3425.
(19) McDonald, C. C.; Phillips, W. D.; Mover, H. F. An Electron Spin Resonance Study of Some Complexes of Iron, Nitric oxide, and Anionic Ligands. *J. Am. Chem. Soc*. **1965**, *87*, 3319−3326.
(20) Costanzo, S.; Menage, S.; Purrello, R.; Bonomo, R. P.; Fontecave, M. Reexamination of the formation of dinitrosyl−iron complexes during reaction of S-nitrosothiols with Fe(II). *Inorg. Chim. Acta* **2001**, *318*, 1−7.
(21) Butler, A. R.; Glidewell, C.; Hyde, A. R.; Walton, J. C. Formation of paramagnetic monomolecular iron nitrosyl complexes from diamagnetic di- and tetranuclear Iron-Sulphur nitrosyls: Characterisation by EPR spectroscopy and study of thiolate and nitrosyl ligand exchange reactions. *Polyhedron* **1985**, *5*, 797−809.
(22) The cysteine-ND_2 adduct should evidence an EPR spectrum with a different pattern; in fact, the D-atom is characterized by a three-line EPR pattern and an hfsc of ca. one-sixth that of the replaced H-atom. Unfortunately, when running experiments in aqueous solution, part of the deuterated cysteine goes back to the unduterated form, and then both species are involved in the reaction.
(23) Grossi, L.; Montevecchi, P. C.; Strazzari, S. Decomposition of Nitrosothiols: Unimolecular versus Autocatalytic Mechanism. *J. Am. Chem. Soc.* **2001**, *123*, 4853−4854.
(24) Grossi, L.; Montevecchi, P. C. A Kinetic Study of *S*-Nitrosothiol Decomposition. *Chem. Eur. J.* **2002**, *8*, 380−387.

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