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Re-examination of the formation of dinitrosyl–iron complexes during reaction of *S*-nitrosothiols with Fe(II)

Simona Costanzo [a], Stephane Ménage [b], Roberto Purrello [a], Raffaele P. Bonomo [a,*1], Marc Fontecave [b,*2]

[a] *Dipartimento di Scienze Chimiche, Università di Catania, Viale A. Doria 6, 95125 Catania, Italy*
[b] *Laboratoire de Chimie et Biochimie des Centres Redox Biologiques, CEA, Grenoble, DBMS/CB, 17 Avenue des Martyrs, F-38054 Grenoble Cedex 9, France*

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Abstract

The reaction of *S*-nitrosothiol compounds with ferrous ions in solution has been investigated and the generated dinitrosyl–iron complexes have been characterized. During the reaction of *S*-nitrosocysteamine with Fe(II) in water solution in the presence of a twofold excess (with respect to iron) of cysteamine hydrochloride (CSH), an EPR-silent dinuclear iron complex (complex A of formula $[Fe_2(RS)_2(NO)_4]$) was formed as the major species and was characterized by FAB MS^+, UV–Vis, NMR and IR spectroscopies. In the presence of a large excess of CSH (CSH/Fe(II) = 20:1), a green paramagnetic mononuclear complex (complex B of formula $[Fe(RS)_2(NO)_2]^-$) was formed. From EPR and UV–Vis data, and also on the basis of the few crystallographic structures known for similar complexes, complex B is proposed to display a distorted tetrahedral geometry (C_{2v}), approaching a trigonal bipyramid with a missing ligand, with the unpaired electron mainly localized on the d_{z^2} orbital of the iron characterized by a d^9 electronic configuration. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Iron complexes; Nitrosyl complexes; Nitrosothiol complexes

1. Introduction

Nitric oxide (NO) is involved in a large number of physiological processes including neurotransmission [1], immune system regulation [2,3], smooth muscle relaxation [4–6] and platelet inhibition [7,8]. The high reactivity of NO with regard to molecular oxygen [9], superoxide anion [10], and heme [11] as well as non-heme iron [12] and the ready availability of these reactants in the plasma and cellular environment suggest that NO is stabilized in vivo by incorporation into a carrier molecule that prolongs its lifetime and preserves its biological activity. In these regards, *S*-nitrosothiols (RSNOs) [14,13] and dinitrosyl–iron complexes (DNICs) [15], have been claimed to subserve the function of transport and storage of NO [16]. RSNOs formed either by low molecular weight thiols or thiol-containing proteins have been detected in vivo [17,18] and were shown to elicit physiological and biochemical responses similar to those elicited by NO [19–21]. The DNICs are formed during the reaction of NO with Fe(II) in the presence of low molecular weight thiols, aminoacids, peptides or proteins (through their cysteine and histidine residues). Such complexes were first observed in biological systems about 30 years ago by their characteristic EPR signal [22–24], but their physiological significance is still a matter of investigation. They have been proposed as endothelium derived relaxing factor (EDRF) candidates [25] and shown to exhibit antiplatelet [26], vasorelaxant [27] and blood pressure lowering activity [28]. Whereas the DNICs bound to protein thiols occur only as paramagnetic forms, DNICs with low molecular weight thiols are known to exist in two forms, paramagnetic and diamagnetic, which are interconvertible [29].

[1] *Corresponding author. Tel.: +39-095-738 5093; fax: +39-095-58 0138; rbonomo@dipchi.unict.it

[2] *Corresponding author. Tel.: +33-4-76-88 9103; fax: +33-4-76-88 9124; mfontecave@cea.fr

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Metal ions have been shown to promote the decomposition of RSNOs [30–33]. Recently we showed that the iron chelators greatly stabilized RSNOs in solution, suggesting that iron ions, as well, were able to react with RSNOs [31]. In this work, we report the results of a study on the solution chemistry of reaction mixtures of *S*-nitrosocysteamine (CSNO) and Fe(II) in water and show that the DNICs, characterized by a variety of spectroscopic methods, are formed spontaneously, in agreement with the preliminary reports [34,35]. This opens the possibility that NO can be transferred from thiols to iron also in biological systems, thus defining RSNOs and DNICs as the two major interconvertible stable biological forms of NO.

2. Experimental

2.1. Materials and methods

Cysteamine hydrochloride and HEPES were purchased from Sigma; $FeSO_4 \cdot 7H_2O$ (99.999%) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) were purchased from Aldrich, *tert*-butyl nitrite (90%) (*t*BuONO) was purchased from Fluka. 1-Amino-2-methylpropane-2-thiol has been synthesized according to Ref. [31]. Milli-Q ultrapure water has been used in all experiments.

Oxyhemoglobin (HbO_2) was prepared from red blood cells according to the procedure described in Ref. [36]. Deoxyhemoglobin (deoxyHb) was obtained, after removing most of the oxygen in solution to avoid side reactions, by adding a slight molar excess of sodium dithionite to a solution of HbO_2 in Tris 20 mM pH 7.5.

CSNO was synthesized in a degassed water solution by reacting 1 M CSH with 1.1 equiv. of *t*BuONO for 15 min at room temperature according to the method described in Ref. [31]. Its concentration was evaluated by measuring the intensity of the absorption band at 333 nm ($\varepsilon = 793$) [31]. Solutions are prepared, kept on ice in the dark and used on the same day. The reaction of CSNO with ferrous iron was investigated during incubation of 2 mM CSNO with 1 mM $FeSO_4 \cdot 7H_2O$ (prepared in deaerated water) in the presence of a twofold and a twentyfold excess of CSH in 0.1 M HEPES buffer pH 7.8 at room temperature. The same results were obtained when the reaction was carried out in water adjusting the pH at 7.8 with NaOH.

2.2. Spectroscopic measurements

Room temperature and frozen solution EPR spectra were performed on a Bruker EMX spectrometer equipped with an X-band bridge ER 041 XG and on a Bruker ER 200 D X-band spectrometer, driven by the ESP 3220 data system, both equipped with a Bruker cryostat for low temperature operations. High field EPR experiments at 285 GHz were performed on acetonitrile solutions of complex B at the temperature of 5 K on a home-built spectrometer [37,38]. The concentration of complex B was measured by the double integration of the EPR signal using a solution of TEMPO of known concentration as a standard. Simulations of the spin hamiltonian parameters were carried out using the Bruker Simphonia program for the room temperature spectra and a program written by Weihe [39] for the frozen solution spectra. In the case of high field EPR spectra, g values were corrected by using the g_{iso} value obtained by the X-band experiments. Instrumental settings are reported in each figure caption associated with the corresponding experimental EPR spectrum.

Absorption spectra were recorded both on a Varian Cary 1-Bio double beam spectrophotometer and on a Hewlett–Packard HP 8452 A diode array spectrophotometer.

^1H NMR spectrum was obtained in D_2O from a Varian Inova operating at 500 MHz.

IR spectra were obtained from on a Bruker Vector 22 FTIR spectrophotometer using KBr pellets.

FAB MS^+ spectral analyses were performed on Kratos MS50 spectrometer equipped with a cesium ion gun operating at 20 kV and interfaced with a MSS data system. 3-Nitrobenzylalcohol has been used as a matrix.

3. Results

The reaction of CSNO with a ferrous salt in water at pH 7.8 was studied and it was found that, under these conditions, two different complexes were obtained, depending on the excess of CSH present in the reaction mixture. At low excess (twofold with respect to iron) a yellow diamagnetic complex (A) was obtained, whereas at large excess (at least twentyfold with respect to Fe), a green paramagnetic complex (B) was the only product of the reaction.

3.1. Spectroscopic properties of complex A

The optical spectrum (250–900 nm) of a reaction mixture containing CSNO, ferrous iron and CSH in a 2:1:2 ratio displayed two bands at 305 and 362 nm, a shoulder at 440 nm and a much less intense band at 755 nm (Fig. 1). Complex A could be precipitated, in the form of a light brown powder, when a concentrated (40 mM Fe) solution of the complex in water was adjusted to pH 7.8 by dropwise addition of NaOH. Dissolution of that powder in water gave the same spectrum (Fig. 1) showing that the great majority of the iron resided in complex A. Unfortunately, it was not possible to further purify the complex for getting crystals.

The FAB MS^+ spectrum showed a $M+1$ peak at 385 m/z (Fig. 2) which clearly indicated that complex A was a dinuclear Fe complex, with two cysteamine moieties and four NO groups, thus with the stoichiometry $Fe_2(CS)_2(NO)_4$. The presence of NO groups is also supported by the appearance of two peaks at 355 ($M+1-NO$) and at 324 ($M+1-H-2NO$), corresponding to two fragments of the molecule lacking one and two NO groups, respectively. The peak at 284 ($M+1-H-NO-SC$) could be due to the loss of a NO group and a cysteamine moiety from the molecular ion, whereas the major peak at 153 m/z can be tentatively assigned to the cysteamine disulfide.

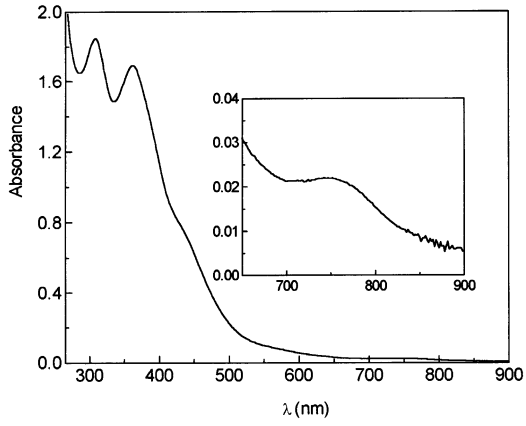

Fig. 1. UV–Vis spectrum of complex A in HEPES buffer (0.1 M, pH 7.8).

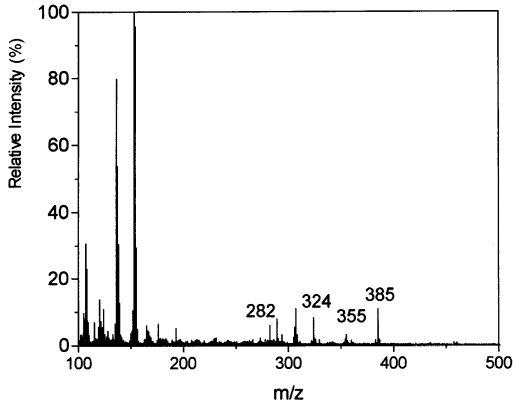

Fig. 2. FAB MS^+ spectrum of complex A in 3-nitrobenzylalcohol.

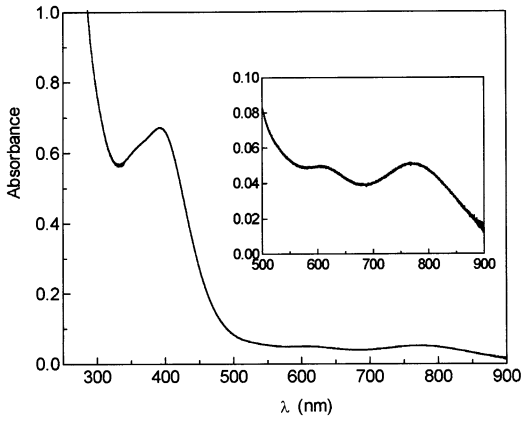

Fig. 3. UV–Vis spectrum of complex B 1 mM in HEPES buffer (0.1 M, pH 7.8).

The IR spectrum of complex A showed two bands at 1770 and 1730 cm^{-1}, corresponding to the symmetric and asymmetric stretching vibrations of the NO ligands, respectively. These values, which are close to those found by Butler et al. [40,41] for $Fe_2(SBut)_2(NO)_4$, do not allow to unambiguously conclude whether nitrosyl ligands should be regarded as NO^+ rather than neutral or negatively charged NO ligands [42,43]. The presence of NO in the complex was further confirmed by the observation that the reaction of complex A with deoxyhemoglobin generated HbNO, the ferrous hemoglobin–NO complex, characterized at 77 K by the typical rhombic EPR signal with a three line hyperfine splitting in the parallel (g_z) region (data not shown).

The ^1H NMR spectrum of complex A in D_2O clearly showed that the cysteamine moieties were bound to the Fe center: two triplets at 3.24 and 3.34 ppm are assigned to the two methylene groups of cysteamine which are shifted downfield with respect to the free thiol (data not shown). The ^1H NMR spectrum also reveals the presence of a unique impurity identified as the cysteamine disulfide.

3.2. Spectroscopic properties of complex B

Complex B is a mononuclear $S=1/2$ paramagnetic species. Quantification of its EPR signal, using TEMPO as a standard, demonstrated that all the iron present in the reaction mixture was incorporated in the paramagnetic complex upon reaction of CSNO with Fe(II) in the presence of a large excess of CSH (twentyfold with respect to iron). Complex B could also be generated from complex A during reaction with 20 equiv. of CSH, as shown from the appearance of the characteristic EPR spectrum at room temperature. Under anaerobic conditions complex B was stable but in the presence of air or hydrogen peroxide it decomposed to generate complex A, as shown by the changes in the light absorption spectrum and from the decay of the EPR signal.

The UV–Vis spectrum (250–900 nm) of a water solution of complex B (Fig. 3) displayed an absorption band at 392 nm ($\varepsilon \approx 3580\ M^{-1}\,cm^{-1}$) and two d–d bands at 603 ($\varepsilon \approx 299\ M^{-1}\,cm^{-1}$) and 772 nm ($\varepsilon \approx 312\ M^{-1}\,cm^{-1}$), responsible for the green color of complex B solutions. The room temperature X-band EPR spectrum (Fig. 4) showed a signal centered at $g_{iso}=2.030$ with a 13-line superhyperfine structure assigned to the interaction of the unpaired electron, primarily localized

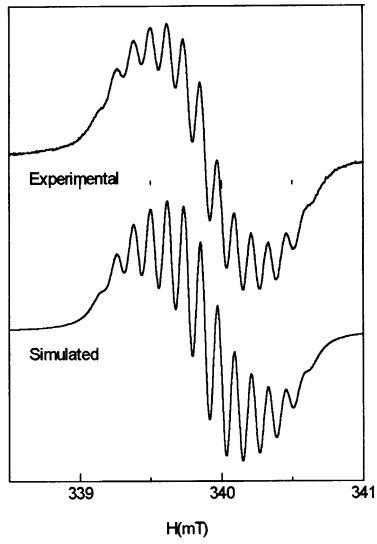

Fig. 4. EPR spectrum at room temperature of complex B. The spectrum has been recorded on a Bruker EMX spectrometer and the instrumental parameters are: microwave frequency, 9.66 GHz; microwave power, 10 mW; receiver gain, 6.32×10^4; modulation frequency, 100 kHz; modulation amplitude, 0.2 G; and time constant, 5.120 ms. The calculated spin hamiltonian parameters are: $g_{iso} = 2.030$, $A_N = 2.35$, and $A_H = 1.21$.

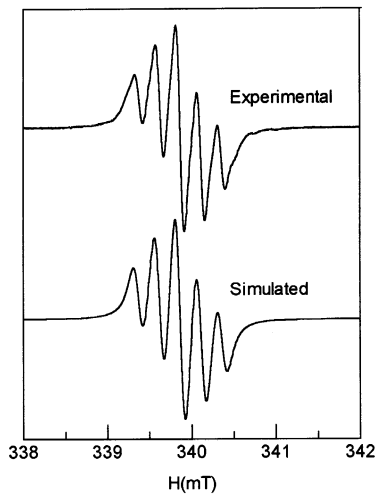

Fig. 5. EPR spectrum at room temperature of the dinitrosyl–iron complex with 1-amino-2-methylpropane-2-thiol. The spectrum has been recorded on a Bruker EMX spectrometer and the instrumental parameters are: microwave frequency, 9.66 GHz; microwave power, 10 mW; receiver gain, 6.32×10^4; modulation frequency, 100 kHz; modulation amplitude, 0.2 G; and time constant, 5.120 ms. The calculated parameters are: $g_{iso} = 2.030$ and $A_N = 2.47$.

on the Fe atom, with the nitrogen atoms of two NO groups and with the four protons of the methylene groups α to the sulfur atoms of the cysteamine moiety, in agreement with the simulated spectrum using $A_N = 2.35$ G and $A_H = 1.21$ G [44]. Accordingly, when CSH was replaced by 1-amino-2-methyl propane-2-thiol during the synthesis of complex B, the resulting EPR signal had only five lines (Fig. 5), reflecting the lack of the hydrogen atoms on the methylene group α to the sulfur. In fact, methyl groups are expected to exhibit very small and probably irresolvable hyperfine interaction. By lowering the temperature the isotropic signal lost its shf structure but persisted down to 255 K, although the solution at that temperature was already frozen (Fig. 6). Further decreasing the temperature, an anisotropic, apparently axial, signal appeared at X-band (Fig. 6) and remained unaltered down to 77 K with $g_\perp = 2.042$ and $g_\parallel = 2.014$, in agreement with the values reported for similar complexes [45]. At high magnetic fields (285 GHz) the EPR signal (Fig. 7) revealed, in fact, that the complex had a rhombic symmetry with $g_z = 2.015$, $g_x = 2.033$, $g_y = 2.041$, thus explaining the broadness of the X-band low field component at 160 K.

4. Discussion

These iron complex species have been previously reported by Vanin et al. [35] but the products of the reaction were not fully characterized. Here, the use of a variety of spectroscopic methods allowed us to identify the complex species in solution coming out from the reaction (UV–Vis as well as mass spectra and high filed EPR spectra are shown for the first time).

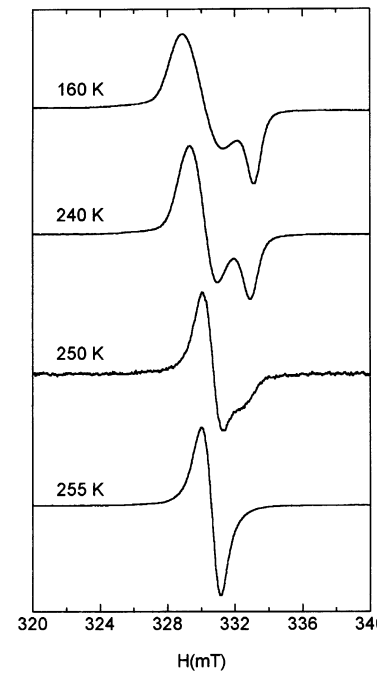

Fig. 6. EPR spectrum at variable temperature of complex B. The spectra have been recorded on a Bruker ER 200 D spectrometer and the instrumental parameters are: microwave frequency, 9.41 GHz; microwave power, 10 mW; receiver gain, 1.00×10^3; modulation frequency, 100 kHz; modulation amplitude, 2 G; and time constant 327 ms.

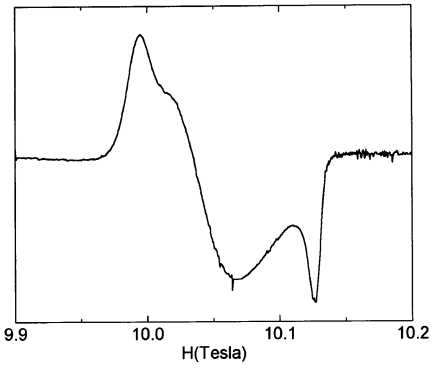

Fig. 7. High field (285 GHz) EPR spectrum of complex B. The spectrum has been recorded on a home-built spectrometer at the temperature of 5 K and the instrumental parameters are: field modulation frequency, 935 Hz; scan rate, 0.5 mT s^{-1}; time constant, 300 ms; and modulation amplitude, 8.55 mA.

One of the complexes, complex B, is a $S = 1/2$ paramagnetic species, as shown by EPR spectroscopy. The EPR signal at room temperature is consistent, also on the basis of the literature [44], with that of a mononuclear iron complex in which Fe is coordinated to two NO molecules and the sulfur atoms of two cysteamine moieties. The complex $[Fe(RS)_2(NO)_2]^-$ has 17 electrons, and its iron-nitrosyl unit can be described with the formal electron assignment $[Fe(NO)_2]^9$ according to the Enemark–Feltham formalism which stresses the well-known covalency and delocalization in the electronically amorphous $Fe(NO)_2$ unit without committing to a formal oxidation state on Fe [46]. On the other hand, EPR and UV–Vis data, taken altogether, are rather consistent with a tetrahedral geometry with a d^9 configuration for the iron atom. Accordingly, the two bands in the UV–Vis spectrum at 603 and 772 nm have ε values higher than those found in octahedral complexes and typical of complexes with a tetrahedral geometry, due to the 'd–p mixing'.

The accurate EHMO calculations made by Summerville and Hoffmann [47] on the $[Fe(NO)_2X_2]^-$ fragment ($X = Cl^-$, I^-), assuming a C_{2v} symmetry, showed that the angle between π acceptor nitrosyl groups was larger than that between the Cl^- or I^- donors. They estimated the N–Fe–N angle to be about 124°, a value which is in agreement with crystallographic data reported in the literature [48–50]. Changing their reference axis system, as considered by Lever [51], simply rotating by 90° and thus interchanging y with z (C_{2v}(III) in Lever's formalism) in the zy plane, the following energy sequence for the d orbitals is found: $2a_1(d_{z^2}) > b_2(d_{yz}) \geq b_1(d_{xy}) > a_2(d_{xz}) > 1a_1(d_{x^2-y^2})$.[3] This reference system is more suitable to describe the electronic features of the $[Fe(NO)_2RS_2]^-$ fragment, whose geometry, as suggested by Briar et al. [45], is better described as a trigonal bipyramid with a missing ligand rather than a simple tetrahedron. In an iron d^9 configuration the HOMO of the complex is the d_{z^2} orbital, which does not point along the directions of the metal–ligand bonds, and, therefore, low superhyperfine nitrogen constants were observed. The odd electron would have small capacity of transferring spin density to the nitrogen nuclei of the donors in the equatorial plane and only a dipolar mechanism should operate. The 13 line EPR signal could be simulated taking into account 4 equiv. hydrogen atoms of methylene groups and 2 equiv. nitrogen atoms of NO groups with quite similar A_{iso} values (1.21–2.35 G). It is not to exclude that the d_{z^2} ground state could receive contribution from the closest levels, whose energies are functions of variations in the N–Fe–N or S–Fe–S angles [47].

Upon freezing the solution, the shf structure in the EPR spectrum disappeared and a reversed pattern $g_\perp$(or g_{xy}) $> g_\parallel$ was observed confirming the structural peculiarities mentioned above, i.e. d_{z^2} as the electronic ground state and the geometry of the complex better described by a trigonal bipyramid with a missing ligand. Although in the case of a trigonal bipyramidal geometry the $g_\parallel$ value is expected to be equal to the free electron value,[4] in C_{2v} symmetry group both $d_{x^2-y^2}$ and d_{z^2} belong to a_1; thus a contribution from $d_{x^2-y^2}$ to the d_{z^2} ground state could explain the greater $g_\parallel$ value (2.014) experimentally obtained. Moreover, by using the rough perturbation formulas given by Goodman and Raynor [52], considering a spin–orbit coupling constant of -201 cm^{-1} (obtained by extrapolating spin–orbit coupling data for iron, reported by Figgis [53]) and an orbital reduction factor of 0.7, a $g_\perp$ value of 2.048 was obtained, assuming $\Delta = 13\,000$ cm^{-1}.[5]

Similar EPR results were recently obtained on solutions of $[Fe(NO)_2(1\text{-methylimidazole})_2]^+$ [50], the crystal structure of which showed a complex pseudo-tetrahedral geometry.

The optical band at 772 nm could, thus, be assigned to the electronic transitions, $^2B_2(d_{yz}) \leftarrow {}^2A_1(d_{z^2})$ and $^2A_2(d_{xy}) \leftarrow {}^2A_1(d_{z^2})$, b_2 and a_2 being the orbitals which can 'mix' via spin–orbit coupling when considering the magnetic field perpendicular to the z-axis. Hence, the observed slight anisotropy in plane could be due to the energy difference between a_2 and b_2. The optical band,

[3] The small discrepancy with Lever's results depends on the fact that his reference axis system was generated starting from the cube axis system.

[4] When the magnetic field is parallel to the z-axis there should be no contribution from mixing with other d orbitals via spin–orbit coupling.

[5] Δ Represents the average energy separation between the orbitals which contains the free electron ($2a_1$,d_{z^2}) and the lower energy orbitals (b_2, d_{xz} and a_2, d_{yz}).

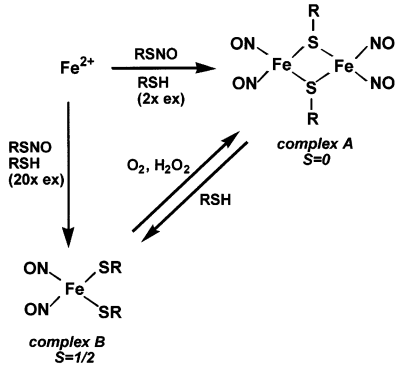

Fig. 8. Proposed structures for complexes A and B.

however, is particularly broad and did not reveal any splitting. The band at 603 nm has to be assigned to $^2A_1(d_{x^2-y^2}) \leftarrow {}^2A_1(d_{z^2})$, which is the other electric dipole allowed transition in C_{2v} symmetry. Moreover, since, in such a dynamic situation, the vibronic mechanism could operate to allow the otherwise forbidden transition and b_1 and b_2 levels have comparable energies, it would be better to assign the electronic transition of the optical band at 772 nm to $^2B_2(d_{yz})$, $^2B_1(d_{xz}) \leftarrow {}^2A_1(d_{z^2})$ (see Fig. 8).

The second complex, complex A, is an EPR-silent dinuclear Fe complex, as shown from mass spectrometry. Its proposed structure, similar to that of the Roussin red salt [54], is shown in Fig. 8. Each iron is coordinated to two NO groups and the two iron atoms are bridged by the sulfur atoms of the two cysteamine molecules. The fact that the complex is EPR-silent suggests that each iron-dinitrosyl unit either has the diamagnetic formal assignment $[Fe(NO)_2]^8$ or the paramagnetic one $[Fe(NO)_2]^9$, as in complex B, with the resulting $S = 0$ state deriving from an antiferromagnetic coupling between the two units. It should be mentioned that complex B generated complex A during reaction with air or H_2O_2 and that A was converted back to complex B in the presence of an excess of cysteamine. One possible explanation, assuming that there is no change in the spin state between A and B and that A has $[Fe(NO)_2]^9$ units, is that A to B conversion is a gain of thiolate, due to the excess of CSH, whereas B to A conversion is a loss of thiolate, consumed by oxidation with O_2 or H_2O_2. Alternatively, A has $[Fe(NO)_2]^8$ units and A to B conversion is due to reduction by excess of CSH, whereas B to A conversion is due to oxidation by O_2 or H_2O_2.

5. Conclusions

We have shown that during reaction of RSNOs with ferrous ions, NO can be transferred quantitatively from the sulfur atom to iron. Depending on the excess of free thiol present in solution, either a paramagnetic or a diamagnetic complex is formed and the two species are interconvertible (Fig. 8). The diamagnetic species is a dinuclear iron-dinitrosyl species in which each iron atom is coordinated to two NO groups and the two iron atoms are bridged by the sulfur atoms of two cysteamine molecules. The paramagnetic species is a 17 electron mononuclear DNIC with a tetrahedral geometry (C_{2v} symmetry group) distorted towards a trigonal bipyramid with a missing axial ligand and a d^9 configuration of the iron atom, with the dz^2 orbital being the HOMO of the complex. The mechanism of NO transfer from the thiol to Fe remains to be investigated. Whether NO is first liberated in solution and rapidly trapped by the metal ions or whether a more concerted and controlled mechanism ($RSNO + Fe \rightarrow RS–Fe–NO$) occurs has to be established.

Acknowledgements

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