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EPR and NMR Studies of Fe(I)–NO Complexes and Their Relevance in Biological and Environmental Processes

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Nitric oxide is implicated in a wide range of biological functions and in many processes of ecological importance. The formation of iron–nitrosyl complexes is fairly well documented, although the oxidation state of the iron atom is not adequately elucidated: the metal ion is in the formal + 1 oxidation state forming low- and high-spin d^7 complexes. Owing to the documented evidence that iron–NO compounds are involved in bioregulatory mechanisms and immune responses, and to the biological relevance of these processes and of related structures, the EPR and NMR features of these complexes were reconsidered and combined EPR and NMR analysis was carried out to obtain information on binding sites and coordination behaviour. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

In December 1992,[1] nitric oxide (NO) was declared 'molecule of the year.' It is a component of smog, a destroyer of ozone, a suspected carcinogen and a precursor of acid rain. On the other hand it plays a role in several physiological processes.[1,2]

Since NO has an unpaired electron, its chemical fate can be studied by EPR spectroscopy. As a free radical, it is very versatile, playing roles in the nervous system, arteries, liver and pancreas and participating in immunochemical and genetic processes, to mention only a few of its functions.

Fe–NO complexes and its paramagnetic properties have been thoroughly investigated by EPR spectroscopy.[2–12]

In view of the importance of nitric oxide in everyday life and the probability of the human body coming into contact with this very reactive molecule, a deeper insight is needed into the interaction between NO and the iron atom and into the change in oxidation state of the metal ion whenever such interaction occurs.

The aim of this paper is to reconsider some EPR results in the light of the loss of intracellular iron in activated macrophage cytotoxicity,[13] the presence of iron–nitrosyl compounds in tissues undergoing rejection,[14] the participation of Fe–NO in the modulation of immune responses[15] and its effects on the natural colours of frescos due to heavy NO pollution in Italy. ESR spectroscopy aided by computer simulation programs was used in order to investigate further the properties of this class of chemical compounds. Combined EPR and NMR analysis was carried out to identify the binding sites and coordination behaviour of the metal complexes.

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EXPERIMENTAL

In order to prepare iron–NO complexes with different ligands, 3–30 mg of the latter were dissolved in water and neutralized ascorbic acid was added up to a final concentration of 30 mg ml^{-1} in a final volume of 1 ml. $FeSO_4$ was added up to a final concentration of 2×10^{-4}–3×10^{-3} M. The pH was adjusted with NaOH or H_2SO_4 and 20 mg of $NaNO_2$ were added and dissolved. As an alternative method, aqueous solutions of $Fe(ClO_4)_2 \cdot 6H_2O$ (5×10^{-4} M) were prepared and saturated with gaseous NO, obtained from $NaNO_2$ by previously reported methods.[16,17] Suitable amounts of ligands were added to the nitrosyliron solutions. The pH was adjusted with NaOH or $HClO_4$.

A sealed Pasteur pipette enclosed in a quartz tube was used for EPR measurements. No differences were observed when flat quartz cells were employed. The solutions were deoxygenated before use by bubbling with nitrogen or by freezing–thawing under vacuum, but no differences were observed when oxygenated solutions were used.

All chemicals were used as received. $Na^{15}NO_2$ was purchased from Aldrich (97% ^{15}N).

EPR spectra were recorded on a Bruker 220D spectrometer with an ASPECT controller on-line,

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equipped with a Bruker variable-temperature accessory. The microwave frequency was 9.4 GHz, the radiation power 20 mW and the modulation amplitude 1.0 G.

Nuclear hyperfine constants were measured by comparison with those of computer-simulated spectra, assuming a purely Lorentzian lineshape. The variables were coupling constants, relative intensities of the hyperfine lines and electron spin relaxation times.

NMR spectra were recorded with a Varian VXR 300-s spectrometer operating at 300 MHz for ^{1}H and at 75 MHz for ^{13}C. The number of transients utilized was 3000 for a total acquisition time of 2 h.

RESULTS AND DISCUSSION

In the metabolism of GTN in mammalian cells, the strong bond between NO and the Fe atom can break the *trans* bond between the Fe atom and the protein, allowing the release of a haem NO moiety.[15] Both haemnitrosyl and non-haem iron–dinitrosyl EPR signals are observed in heart allograft tissue; the formation of these signals is a specific result of immune activation.[13,14] All these findings provide evidence of the role of the iron–nitrosyl complexes in the molecular mechanism of NO action, as reported by Stamler *et al.*[3]

Previous EPR studies[6–10] have determined the following chemical properties of iron–nitrosyl complexes:

(a) Iron is in the formal +1 oxidation state; hence we are concerned with low- and high-spin d^7 complexes as shown by infrared studies and magnetic susceptibility measurements.[18] Fe(I) is very unusual in biological systems and the +1 oxidation state is an interesting feature of this class of compounds that may explain the peculiar role of the NO–iron interaction.

(b) Dinitrosyl low-spin d^7 complexes[19] show that the iron atom has a low degree of covalency, with the degeneracy of the d orbitals completely destroyed. The NO groups are present as free radicals, with their unpaired electrons coupled in a molecular orbital. The unpaired spin density is primarily localized on the iron atom as shown by ^{57}Fe experiments[4] that reveal a very high hyperfine coupling constant of 1.56 mT.

(c) All the EPR spectra with small molecules usually have a well resolved superhyperfine structure due to a relatively long electron spin relaxation time. The Fermi contact interaction between the unpaired electron of the iron atom and the ligand nuclei gives direct information on the characteristics and number of the sites involved binding.

(d) Dynamic equilibria exist in solution, giving rise to the simultaneous presence of different chemical species; formation of ternary complexes has also been reported.[7]

Let us recall that, as clearly stated by Stamler *et al.*,[3] the biochemical pathways of nitric oxide share two common features: the enzymatic synthesis of NO from L-arginine and the formation of iron–nitrosyl complexes. They also emphasized that the paramagnetism of these complexes is a composite property of both the metal and ligand electrons. They proposed an extended paradigm in which the NO processes are clearly described. These processes involve L-arginine,[13,14] ADP and iron–dinitrosyl compounds in different pathways. The reduction of Fe(III) is required is some of the biochemical processes.

We suggest that reduction can proceed further to Fe(I), making it of interest to characterize iron–nitrosyl complexes with iron in the formal +1 oxidation state.

Two possible reduction mechanisms may be suggested:

$$2Fe^{2+} \rightleftharpoons Fe^{3+} + Fe^{+}$$

$$Fe^{2+} + 3NO \rightleftharpoons [Fe(NO)_2]^{+} + NO^{+}$$

We have observed that the formation of a mononitrosyl complex is often followed a few minutes later by the appearance of a new spectrum corresponding to a dinitrosyl compound. Obviously redox processes must be involved in this equilibrium.

Owing to the versatile action of nitric oxide towards various organic binding sites and the iron atom (haem or non-haem), the characterization of the physicochemical properties of iron–nitrosyl complexes with L-arginine, ADP and ATP can be useful in the study of the extended paradigm of NO biochemistry. Accordingly, EPR and NMR studies of this type of iron–nitrosyl complex were performed, focusing attention on the paramagnetic properties of the metal and ligand electrons, on the various binding sites and on the possible simultaneous presence of different chemical species in solution.

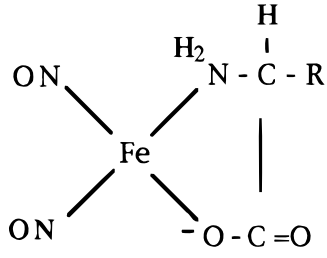

Figure 1 shows the experimental EPR spectra of the $Fe^{I}(NO)_2$ complexes formed with (a) serine and (b) L-arginine. The two spectra are identical and can be explained, for both serine and arginine, in terms of the chelate structure shown, which involves the amino and carboxyl groups of the α-amino acid. The spectrum is not affected by the specific R group of the amino acid.

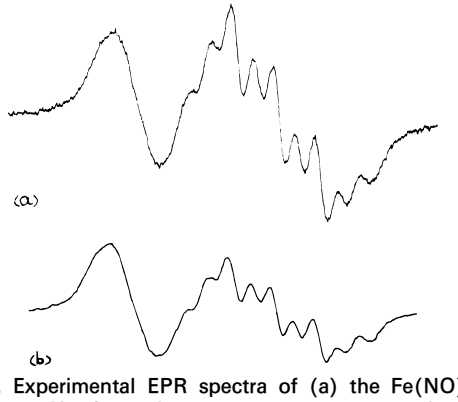

Figure 1. Experimental EPR spectra of (a) the $Fe(NO)_2$–serine system at pH 6 and room temperature and (b) the $Fe(NO)_2$–arginine system at pH 6.6 and room temperature ($g = 2.020$).

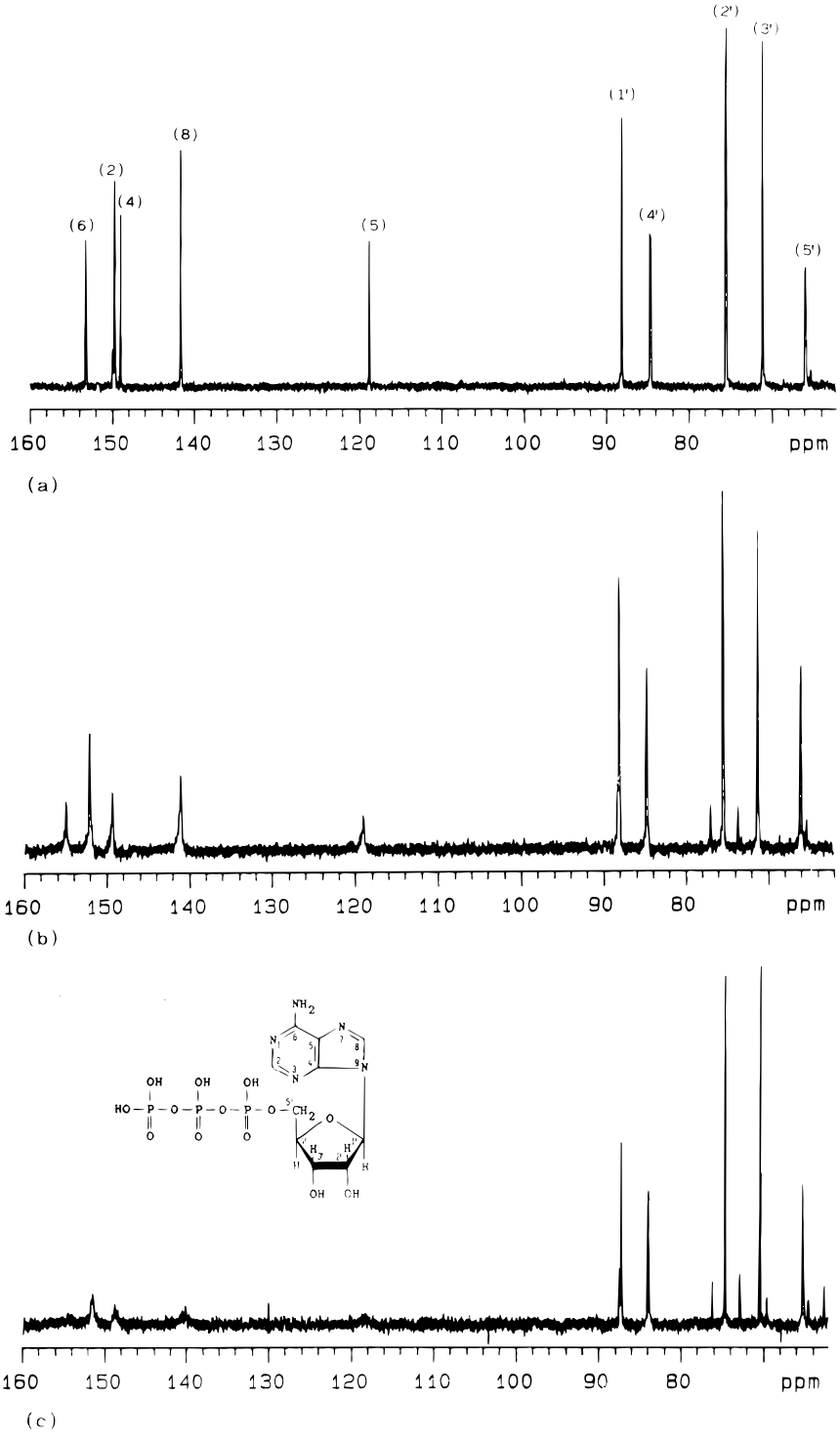

Figure 2. Experimental ^{13}C NMR spectra of (a) ADP 0.2 mol dm^{-3} in D_2O, (b) $Fe(NO)_2$–ADP system [ADP 0.2 mol dm^{-3} and $Fe(NO)_2$ 1×10^{-4} mol dm^{-3}] and (c) $Fe(NO)_2$–ADP system [ADP 0.2 mol dm^{-3} and $Fe(NO)_2$ 5×10^{-4} mol dm^{-3}] at room temperature.

The L-arginine side-chain is not involved. In fact, the nine-line spectrum (with relative intensities of 1:2:4:4:5:4:4:2:1) is consistent with computer-simulated curves constructed on the basis of the proposed structure and hyperfine splitting constants $a_{^{14}NH_2} = 0.66$ mT and $a_{^{14}NO} = 0.34$ mT.

^{15}N isotopic substitution gives a five-line pattern (with relative intensities of 1:3:4:3:1), thus confirming the above findings. The ^{15}N coupling constant is in perfect agreement with the ratio between nuclear g values of 0.71. The computer simulation in the case of the isotopic substitution was carried out assuming the ratio $\mu(^{14}N)/\mu(^{15}N) = 0.71$.

In order to elucidate the molecular structure of the Fe(I)–NO complexes with ADP, phosphate and adenine EPR spectra were recorded and, in the case of phosphate, compared with literature data.[7]

The $Fe(NO)_2$ phosphate complex gives rise[7] to a septet at $g = 2.033$ with coupling constants $a_{^{14}NO} = 0.24$ mT and $a_{^{31}P} = 0.30$ mT and, in the presence of

$Na^{15}NO_2$, to a quintet with $a_{^{15}NO} = 0.34$ mT and $a_{^{31}P} = 0.30$ mT. The $Fe(NO_2$–adenine complex gives rise to a nine-line spectrum at $g = 2.027$ with coupling constants $a_{^{14}NO} = 0.20$ mT and $a_{N(adenine)} = 0.27$ mT and, in the presence of $Na^{15}NO_2$, to a septet with $a_{^{15}NO} = 0.28$ mT and $a_{N(adenine)} = 0.27$ mT. Also in this case the ^{15}N coupling constants are in perfect agreement with the ratio between the nuclear g values and the computer simulation for the isotopic substitution was carried out assuming the ratio $\mu(^{14}N)/\mu(^{15}N) = 0.71$.

The EPR spectra shown by the Fe(I)–NO–ADP complex are similar to the EPR signal of adenine, even if not so well resolved. Comparison with the simulated spectra indicates a complex with two NO molecules and two equivalent base nitrogens bonded to the iron atom, that is, two adenine rings involved in metal coordination, excluding the possibility of chelation. The ^{15}NO spectrum is a septet as expected with two ^{15}NO groups, with apparent relative intensities of 1:4:8:10:8:4:1; agreement with the simulated spectrum is fairly good, assuming $a_{^{15}NO} = 0.28$ mT, $a_N = 0.27$ mT and $\Delta H = 0.23$ mT, in agreement with the ratio between the nuclear g-factors. Although not conclusive proof, this implies that the adenine ring is involved in metal coordination via imidazole nitrogens.

In order to obtain a deeper insight into the metal binding sites and the dynamic situation in solution, the EPR study was combined with NMR analysis. Figure 2 shows the ^{13}C FT-NMR spectra of ADP (a) in D_2O and (b, c) in D_2O in the presence of the iron–nitrosyl complex. The paramagnetic d^7 species with a large unpaired electron spin density on the iron atom greatly affects the ^{13}C nuclear relaxation rates. The selective broadening measurements, performed under several experimental conditions (relative concentrations, pH, temperature), give direct evidence of the binding sites of the iron–nitrosyl group. The disappearance of the C-5 peak and the broadening of the C-8, C-6 and C-4 peaks [see Fig. 2(b) and (c)] strongly suggest direct binding to the imidazole N-7 in the adenine ring.

The same spectra, with a small addition of the iron–nitrosyl complex, display substantial broadening of the C-4′ and C-5′ peaks and weak broadening of the C-2 peak. Further addition of the iron–nitrosyl group gives rise to the situation described above (C-5, C-8, C-6 and C-4 affected), whereas a large addition of the paramagnetic species leads to the disappearance of all ^{13}C peaks of the adenine ring, leaving the other carbon atoms unaffected.

These findings can be explained in terms of the existence of different binding sites in the ADP molecule, assuming the following two-step equilibrium in solution:

$$Fe^{I}(NO)_2 + ADP \rightleftharpoons Fe^{I}(NO)_2ADP_{phosphate} \rightleftharpoons Fe^{I}(NO)_2ADP_{phosphate+N\text{-}7}$$

With a small concentration of $Fe(NO)_2$ only the phosphate group is involved in the metal binding. On adding more $Fe(NO)_2$, the equilibrium is shifted towards the formation of a complex involving N-7 in the adenine ring as a binding site. In this case either a form with two ADP molecules bonded to the metal ion through N-7 or a back-bound phosphate–metal ion–ring interaction, as suggested in the proposed equilibrium, are consistent with the above findings.

^1H FT-NMR spectra confirm the assumption of the involvement of N-7 and the two-step equilibrium in solution. In fact, the proton relaxation time of H-8 is the most affected by the paramagnetic interaction. At low concentrations of $Fe(NO)_2$ the peaks of H-3′ and H-4′ are slightly broadened.

The ^{31}P NMR spectra, performed under similar experimental conditions, do not provide any further information in terms of structural features, as the α and β peaks do not show substantial differences.

In conclusion, combined EPR and NMR analysis is very important when different binding sites play competitive roles and equilibria between different chemical species occur, in order to give structural assignments and to show the presence of multiple species in solution.

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