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Nitric oxide, a biological effector

Electron paramagnetic resonance detection of nitrosyl-iron-protein complexes in whole cells *

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Abstract. Nitric oxide has been used for more than 20 years as an electron paramagnetic resonance probe of oxygen binding sites in oxygen-carriers and oxygen-metabolizing metalloenzymes. The high reactivity of NO with oxygen and the superoxide anion and its high affinity for metalloproteins led biochemists to consider NO as a highly toxic compound for a living cell. This assertion has recently been reconsidered following a number of discoveries of great significance: the finding of the activation of guanylate cyclase by NO, the recognition that NO is the precursor of nitrite and nitrate ions released in the activation of macrophages by endotoxin and cytokines, evidence that NO is an Endothelium-Derived Relaxing Factor, and the discovery of NO-biosynthesis from L-arginine, a pathway common in various biological cell-to-cell signalling processes. It is now admitted that NO plays a key bioregulatory role within mammalian cells, between cells of different types and in the host defence response. In the present review we have attempted to give a general picture of what is known of the chemical, physical, biochemical and biophysical properties of NO among the various nitrogen oxides. We have focussed on the structural information that can be obtained by electron paramagnetic resonance spectroscopy of nitrosyl-metalloprotein complexes. Finally we have shown how molecular targets of nitric oxide can be characterized, within whole cells, by electron paramagnetic resonance spectroscopy.

Key words: Nitric oxide – Nitrogen oxides – Electron paramagnetic resonance – Macrophage – Glyceryl trinitrate

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Abbreviations: BCG: Bacillus Calmette-Guérin; CcO: cytochrome *c* oxidase; cGMP: cyclic GMP; Cyt. cd_1: cytochrome cd_1 or nitrite reductase from *Pseudomonas aeruginosa;* DPG: 2,3-diphosphoglycerate; EDRF: endothelium-derived relaxing factor; EPR: electron paramagnetic resonance; GC: guanylate cyclase; GMN, GDN, GTN: glyceryl mono-, di-, trinitrate; GSH, GSSG: reduced and oxidized glutathione; GSH-ST: glutathione *S*-transferase; Hb: hemoglobin; Hb^{3+}: ferrihemoglobin; IFN-γ: interferon gamma; IHP: inositol hexaphosphate; LPS: lipopolysaccharide from *E. coli;* Mb: myoglobin; NMMA: N^G-monomethyl-L-arginine; P-450: cytochrome P-450; P-420: cytochrome P-420; P1, P2, P3, P7: isoperoxidases from turnip; SHF: superhyperfine structure; TDO: tryptophan 2,3-dioxygenase from *Pseudomonas fluorescens;* TNF: tumor necrosis factor

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Introduction

The recent discovery that mammalian cells synthesize inorganic nitrogen oxides from L-arginine has resulted from three originally independent lines of research: studies of macrophages as a cytotoxic effector cell, studies of endothelium-derived vascular relaxation and studies of the pro-carcinogenicity of nitrogen oxides. In less than five years, a large and colorful jigsaw puzzle has rapidly been assembled (Fig. 1).

First discovered in macrophages (see the reviews: Marletta 1988, 1989; Hibbs et al. 1990), and in endothelial cells (see the reviews: Ignarro 1989, 1990; Moncada et al. 1989), as shall be discussed further, the L-arginine dependent synthesis of nitric oxide also has detectable effects on many target cells or tissues. The best known instances are tumor cells co-cultivated with macrophages (Hibbs et al. 1987 a, b; Stuehr and Nathan 1989) or aortic smooth muscle strips or platelets for endothelial cells (Palmer et al. 1987; Sneddon and Vane 1988; Moncada et al. 1988). The synthesis of NO in the generator cell induces an increase of cGMP levels both in the generator and in the target cells (Moncada et al. 1988, 1989). Synthesis has been detected in other tissues such as neutrophils, *T* lymphocytes, adrenal tissue and cerebellar tissue. Similar cell to cell responses, or transcellular signalling were found in the central nervous system (Knowles et al. 1989; Moncada et al. 1989; Gally et al. 1990), in the innervation

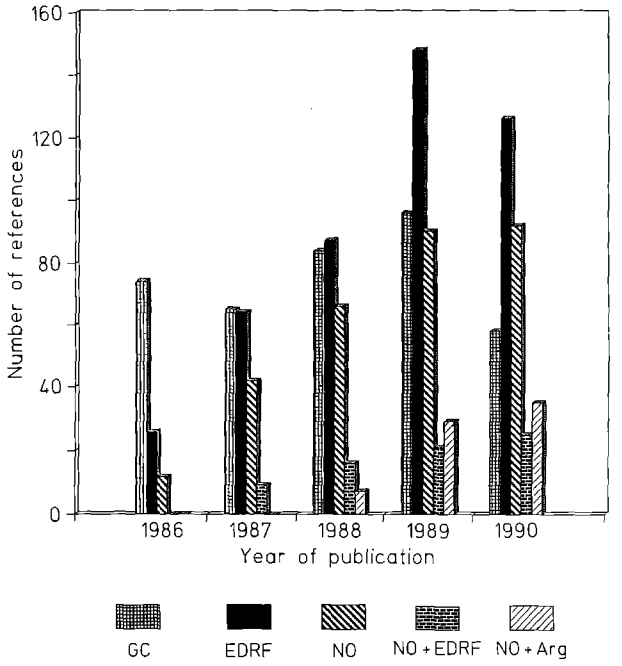


Fig. 1. Number of references as a function of the year of publication as found by bibliographical research (January 30, 1991) using Medline as the data base. Three index key words were used, nitric oxide, EDRF and guanylate cyclase, and the combination NO + EDRF and NO + arginine. *Comments: 1*) The number of references scored in 1990 is greatly underestimated, perhaps by as much as 100%, because of a four month lag in entering references. *2*) The relatively constant number for GC corresponds to the fact that the enzyme was discovered in 1969 and its activation by NO in 1977. *3*) The already high number of articles for NO in 1986 (12) and 1987 (42), before the discovery that NO was an EDRF in 1987, may be explained by the great interest in NO as an intermediate in denitrification. *4*) The bioregulatory role of L-arginine-derived NO is expressed by the number of papers scored by the combinations NO + EDRF (16 in 1988, 21 and 1989, 25 in 1990) and NO + arginine (7 in 1988, 29 in 1989 and 35 in 1990). *5*) The number of references used in the present review corresponds to 10 to 30% of the number of references indexed by Medline. However for the last three years we have here cited 60% to 90% of these Medline indexed references

of the gastrointestinal tract (Bult et al. 1990; Hata et al. 1990), between hepatocytes and Kupffer cells (Billiar et al. 1989; Curran et al. 1989), in the macrophage-induced biostatic effect on the fungus *Cryptococcus neoformans* (Granger et al. 1988, 1990), on the widespread obligate intracellular protozoan *Toxoplasma gondii* (Adams et al. 1990), in the macrophage killing of the monocyte protozoal parasite *Leishmania major* (Green et al. 1990; Liew et al. 1990), etc. NO synthesis has also been characterized in host defence mechanisms, such as the case of septic shock (Hutcheson et al. 1990; Kilbourn et al. 1990; Knowles et al. 1990). NO synthase has been localized and associated with selective and discrete neuronal populations (Bredt et al. 1990). While the NO synthesis pathway is apparently the same in these different cellular types, it is presently clear that there are at least two types of NO synthases. One is constitutive, in the brain, endothelial cells, platelets, neutrophils, adrenal; the other is inducible by endotoxin and/or cytokines, and is also found in liver and lung (Knowles et al. 1990). NO synthase, always NADPH-dependent, appears to have variable co-factor requirements depending upon its source (Tayeh and Marletta 1989; Kwon et al. 1989). Its dependence upon Ca^{2+} and calmodulin is certainly quite variable (Marletta 1989; Bredt and Snyder 1990; Knowles et al. 1990). For instance, while NO synthesis by macrophages lags several hours after IFN and/or LPS stimulation, it occurs as a burst in the case of bradykinin stimulation of endothelial cells. A large amount of research is presently underway in many laboratories in order to characterize the enzymological status of NO synthase originating from various cellular types. The role of this pathway in widely different pathological states such as atherosclerosis, septic shock, chronic inflammation and in carcinogenesis is unknown, but certainly of great importance.

In order to understand the vast biological implications of this newly discovered L-arginine-dependent pathway of nitric oxide synthesis, it can be useful to summarize (Sect. 1) the long-known relevant chemical properties of the various nitrogen oxides in water solution. Secondly, a synthetic review (Sect. 2 and 3) of the endogeneous or exogeneous origin of the nitrogen oxides and of their toxicity in mammalian cells shall be attempted and compared to what is known of the nitrogen cycle in microbiology.

In the case of nitric oxide, the complexes formed with numerous metalloproteins and enzymes have been characterized, mostly by electron paramagnetic resonance spectroscopy. A useful excerpt of the wide "dictionary" of the known EPR spectra of nitrosyl-metalloproteins will be presented (Sect. 4).

These chemical, biochemical and biophysical introductions will serve as a background to an overview (Sect. 5) of the recent developments of research on the role of nitric oxide as a biological effector and a regulator in mammalian cells.

Finally two sets of recent EPR experiments shall be presented (Sects. 6 and 7). These experiments were attempted to arrive at a precise characterization of nitric oxide molecular targets in living cells.

1. Summary of the chemistry of nitrogen oxides in water solution

Nitrogen forms oxides and oxidized compounds, which have an oxidation number between +5 and +1, and compounds with a negative oxidation number such as hydroxylamine NH_2OH (−1), hydrazine N_2H_2 (−2) and ammonia (−3). There exist compounds in which two nitrogen atoms have non-equivalent oxidation numbers, such as oxyhyponitrite or trioxodinitrate ($HN_2O_3^-$), nitramide (NH_2-NO_2), imidonitric acid NH = NO(OH) (Averill and Tiedje 1982; Doyle and Mahapatro 1984; Doyle et al. 1988; Weeg-Aerssens et al. 1988).

Nitric anhydride, N_2O_5 and nitrate, NO_3^- are powerful oxidants ($E'_7 = 0.43$ V for NO_3^-/NO_2^-) while nitrous anhydride, N_2O_3 and nitrite, NO_2^- can be either oxidant ($E'_7 = +0.35$ V for NO_2^-/NO) or reducer ($E'_7 = +0.88$ V for N_2O_4/NO_2^-). The nitrite ion cannot dismutate at neutral pH. The nitrosonium cation NO^+ has the same oxidation number as NO_2^-: +3. Nitrogen tetra-oxide, N_2O_4,

produced in the reaction of nitric oxide with molecular oxygen, dismutates spontaneously in water solution yielding nitric acid HNO_3 and nitrous acid HNO_2. It is a diamagnetic dimer of paramagnetic nitrogen dioxide or peroxide NO_2.

Nitric oxide, NO can be an oxidant ($E'_7 = +1.18$ V for NO/N_2O) or a reducer ($E'_7 = +0.35$ V for NO_2^-/NO), but does not dismutate, except when it is coordinated to transition metals (McCleverty 1979). It is paramagnetic. It is a ligand of transition metals, Fe, Cu, Co, Mn, etc, creating a medium to strong ligand field in compounds such as diamagnetic nitroprusside $[Fe^{III}(CN)_5NO]^{2-}$ (Swineheart 1967), paramagnetic $[Fe^{II}(CN)_5NO]^{3-}$ or $[Fe^{II}(H_2O)_5NO]^{2+}$ (Beinert et al. 1965; McDonald et al. 1965; McNeil et al. 1965; Gans 1967; Van Voorst and Hemmerich 1966; McCleverty 1979). However it is rarely placed in the nephelauxetic or the spectrochemical series as are halides or $-CO_2^-$, H_2O, NH_3, OH^- or CN^-. Depending upon the stereochemistry of the complex. NO can be coordinated either as a $(NO)^+$ or as a $(NO)^-$ ligand (Enemark and Feltham 1972; McCleverty 1979).

Finally, although nitrous oxide N_2O has a high thermodynamic potential for reduction to N_2 ($E'_7 = +1.35$ V for N_2O/N_2), it is chemically relatively inert in aqueous solution, acting as a poor oxidant and undergoing no dismutation. The corresponding anion is (*cis* or *trans*) hyponitrite, $N_2O_2^{2-}$. There exists also, with the same oxidation number (+1) the compound nitroxyl H−N=O (Averill and Tiedje 1982; Doyle et al. 1988).

2. Nitrogen oxides in the nitrogen cycle

Nitrogen fixation in plants, algae and bacteria (free living or symbiotic, e.g. *Rhizobium* in leguminous root nodules, aerobic such as *Azotobacter*, anaerobic such as *Clostridium pasteurianum, Klebsiella pneumoniae* in the intestine flora, organotropic or photolithotrophic such as *Chromatium, Rhodospirillum*) allows the enzymatic reduction of N_2, a chemically inert gas, to ammonia (Quispel 1974; Burns and Hardy 1975; Brock 1979), catalyzed by nitrogenase. N_2O is an alternative substrate for nitrogenase (Vincent et al. 1981). Ammonia is enzymatically immobilized as amino-acids or derivatives (glutamic acid, glutamine, etc).

Ammonia is also oxidized by autotrophic aerobic nitrifying bacteria, widespread in soil and water, to hydroxylamine and nitrate (*Nitrosomonas*), N_2O and NO being produced as intermediates. Similarly, nitrite is oxidized to nitrate (*Nitrobacter*).

Nitrate and nitrite are in turn reduced by two separate microbiological processes: the assimilatory reduction of nitrate to ammonia by *Azotobacter, Aspergillus, Neurospora*, etc. (Guerrero et al. 1981) and denitrification, a gas-producing reduction of nitrate, nitrite and N_2O by anaerobes and facultative anaerobes (*Pseudomonas, Paracoccus*, etc) (Payne 1981; Bessières and Henry 1984; Henry and Bessières 1984; Aerssens et al. 1986; Ferguson 1987; Weeg-Aerssens et al. 1988). The gas products can be, depending upon the bacteria, NO (rarely), N_2O or N_2 (most often). Denitrification is properly a respiration process upon nitrate or nitrite; it is also called nitrate-dissimilation.

All these redox reactions from nitrate to ammonia and vice-versa are catalyzed in cellular bioenergetic processes (Zumft and Cardenas 1979) by enzymes which are all metalloproteins containing multiple metal sites: hemoproteins containing hemes *a*, *b*, *c* or *d*, Mo and FeS containing proteins, copper proteins, copper and heme containing proteins, etc.

3. Exogeneous character and toxicity of nitrogen oxides in mammalian cells

In mammals, the general metabolism of nitrogen oxides is poorly understood. Nitrate and nitrite have long been thought to be exclusively of exogeneous origin. In fact, nitrate arises mostly in water from fertilizers and pesticides and is normally excreted in urines. The maximal norms are 50 mg per liter (≈ 1 m*M*) in Europe, 0.73 m*M* in the USA; sometimes greatly exceeded, especially in agricultural regions. Nitrite is itself added for meat conservation or "meat-curing", where it has antioxidant, antimicrobial and antibotulinal properties (Bonnet et al. 1980; Reddy et al. 1983). Other major sources of NO, NO_2, NO_2^- and NO_3^- are motorcar and aircraft exhaust fumes, industrial smokes and cigarette smoke.

The main aspect of nitrate and nitrite toxicity is their pro-carcinogenicity, leading through reactions with secondary amines to carcinogenic *N*-nitrosamines RR′NNO (Iqbal et al. 1980; Tannenbaum 1987; Challis et al. 1987; Mirvish et al. 1987).

It is now known that nitrate and nitrite are endogeneous cellular components in many types of mammalian cells. It is trivial to say that the degree of toxicity of these anions and gases is related to concentrations, cellular compartmentation, relative affinity for various binding sites, relative kinetics of reduction, etc, all factors that still need to be measured.

In humans, NO_3^- is reduced to NO_2^- by bacteria in the oral cavity, while NO_2^- is reduced to N_2 in the stomach and NO_3^- to NO_2^- and NH_3 by the facultative anaerobic intestinal flora (Hong et al. 1980; Yoshida and Kasama 1987). All these intestinal reduction products are reabsorbed into the blood and thus may be toxic.

A well known effect of nitrite is the autocatalytic oxidation of HbO_2 to Hb^{3+} (Doyle et al. 1985; Spagnuolo et al. 1987; Doyle et al. 1988; Kosaka et al. 1989), yielding also NO_2 and H_2O_2. Furthermore, excess nitrite binds weakly to Hb (Fe^{3+}). While methemoglobin itself is enzymatically reduced to deoxyhemoglobin by erythrocyte methemoglobin reductase, the other two products, NO_2 and H_2O_2, are highly destructive oxidants. Hydrogen peroxide is dealt with by erythrocyte catalase, but NO_2 leads to the formation of nitrosamines (Iqbal et al. 1980). High levels of nitrate in blood are also correlated with methemoglobinemia (over 1% Hb^{3+} in red cells) (Hegesh and Shiloa 1982).

The paramagnetic oxides NO and NO_2 are detected in smokes, cigarette smoke in particular (Pryor et al. 1983; Halliwell and Gutteridge 1985). The toxicity of NO

may arise from several reactions. Firstly NO reacts with O_2 yielding NO_2 and in turn NO_2^- and NO_3^-. NO reacts also with the superoxide anion $O_2^- \cdot$ and its acidic form $HO_2 \cdot$ to give the peroxonitrite $ONOO^-$, that rearranges into nitrate (Blough and Zafirion 1985), apparently producing the hydroxyl radical, HO · (Beckman et al. 1990). Furthermore, both NO and NO_2 react with H_2O_2, present at high concentration in pulmonary alveolar macrophages, to give rise to NO_2^-, or NO_3^-, and the highly reactive hydroxyl radical HO · (Pryor et al. 1983; Halliwell and Gutteridge 1985).

The second reaction is the well studied binding to deoxyhemoglobin. The affinity of NO for Hb, measured at half-saturation, is $3 \times 10^{10}\ M^{-1}$ while those of CO and O_2 are respectively 2×10^7 and $6 \times 10^4\ M^{-1}$. The main reason for such a high affinity is the very slow dissociation of NO from HbNO (mean $t_{1/2} \sim 3$ h) (Antonini et al. 1966; Sharma and Ranney 1978) as compared to that of CO from HbCO (mean $t_{1/2} \sim 35$ s) and O_2 from HbO_2 (~ 20 ms) (Gray and Gibson 1971). It seems that exposure of animals to low levels of nitric oxide (<40 ppm) and nitrogen dioxide leads to low concentrations of HbNO and Hb^{3+} in the blood (Oda et al. 1975; 1980; Yoshida and Kasama 1987). Finally HbNO reacts with O_2 to give rise to Hb^{3+}. Another reaction of NO is that it can bind loosely to Hb^{3+} and reduce it slowly to HbNO (Keilin and Hartree 1937; Sancier et al. 1962; Chien 1969 a; Addison and Stephanos 1986). This reduction reaction of ferric hemoproteins (myoglobin, catalase, peroxidase, etc) can be coupled with an NO attack on nucleophiles such as proline, phenol, *N*-acetylcysteine, to yield respectively *N*-nitrosoproline, nitrosophenol, *S*-nitroso-*N*-acetylcysteine (Wade and Castro 1990). This generation of carcinogenic nitroso derivatives could be of great importance due to the release of NO that might occur in cases of chronic inflammation. Another nitrogen oxide, trioxodinitrate $HN_2O_3^-$ reacts also with Hb^{2+}, HbO_2 and Hb^{3+} (Doyle et al. 1988) giving in turn HNO, NO and NO_2^-.

As exemplified extensively in the next two sections (Sects. 4 and 5), NO has numerous other potential cellular binding sites. In many cases, NO binds at an oxygen binding site and therefore has an inhibitory and thus toxic cellular effect. In other cases, however, the binding has highly regulatory role in cells and organs.

Finally, although it is outside the scope of the present review, let us note that nitrous oxide N_2O is admittedly one of the safest and least toxic of inhaled anaesthetics. It has long been considered chemically inert in the body. In fact it is metabolized in man by the intestinal content (Hong et al. 1980). A copper nitrous oxide reductase has been recognized in several types of *Pseudomonas* (Zumft and Matsubara 1982; Riester et al. 1989). Nitrous oxide has however some toxic properties affecting respiration, circulation and neuronal activity. Both the anaesthetic effect and the side effects of N_2O can perhaps be related to its recently discovered inhibitory effect on mitochondrial respiration (Sowa et al. 1987). Cytochrome *c* oxidase activity is partially and reversibly inhibited by N_2O through binding to three sites of variable polarity away from the hemes, other than the O_2 reaction site, reducing the rate of electron transfer from cytochrome *c* to the oxidase rather than interfering directly with the reduction of O_2 to water (Einarsdottir and Caughey 1988). Independently of the above effect, as explained in Sect. 4.4, NO can be reduced to N_2O at a metal-containing active site of laccase, deoxyhemocyanin, reduced cytochrome *c* oxidase, as it does with nitrite reductase (cytochrome cd_1) from *Pseudomonas aeruginosa*.

4. Nitric oxide, a paramagnetic analogue of molecular oxygen, used as an EPR probe of metalloproteins

4.1. Nitrosyl hemoglobin

Since the early EPR works of Kon (1968), Chien (1969 b), Shiga et al. (1969), nitric oxide has been widely used as a spectroscopic probe of hemoglobin structure, linked to its binding properties (Rein et al. 1972; Trittelvitz et al. 1972; Henry and Banerjee 1973; Henry and Cassoly 1973; Sharma and Ranney 1978; Taketa et al. 1978). The EPR work of Kon and Katoaka (1969) on model compounds and of Yonetani et al. (1972) on peroxidases allowed the complete assignment of the 77 K EPR spectra of nitrosyl hemoproteins: the unpaired electron is little associated with the NO nitrogen atom; the unpaired electron orbital is the $(d_{z^2}) - \sigma^*$ orbital, with a strong $d_\pi - p_\pi$ interaction of the proximal base π and the iron d_π (d_{xz}, d_{yz}) electrons. The 77 K EPR spectra of HbNO and of its subunits αNO and βNO are characterized by three *g*-values g_x, g_y, g_z (Fig. 2 and Table 1). A superhyperfine structure (SHF) of nine lines (triplet of a triplet) is detectable, arising from an interaction of the unpaired electron with two ^{14}N atoms, one from NO ($A_z = 19$ to 21 G), one from an N atom of the proximal histidine (F8) ($A_z = 6.5$ G). In some cases, upon changes of quaternary and tertiary structures induced by modifications of homotropic interactions (Henry and Banerjee 1973), by addition of allosteric effectors (DPG, ATP, IHP) or by pH changes (Rein et al. 1972; Trittelvitz et al. 1972; Henry and Banerjee 1973), this SHF structure changes from a nine line to a three line pattern resulting from the interaction with NO alone ($A_z = 16$ G). In relationship to the bioregulatory role of NO (see Sects. 5 and 6), one of the most important findings, based on EPR and infra-red data, was that the shift from the quaternary R (relaxed) (oxy) structure characterized by the nine SHF lines and the type C in Kon and Kataoka classification, to the T (tense) (deoxy) structure characterized by the three SHF lines and the type B, induced a loosening or even a cleavage of the bond between the proximal histidine (F8) nitrogen atom and the nitrosyl-heme iron atom, at least in α chains (Kon 1975; Maxwell and Caughey 1976; Szabo and Perutz 1976; Sharma and Ranney 1978; Taketa et al. 1978; Scholler et al. 1979), making possible the release of the relatively stable heme-NO moiety for a competitive binding.

4.2. Other nitrosylhemoproteins

Nitric oxide is a high affinity inhibitor of many oxidases and oxygenases through its binding, in place of oxygen,

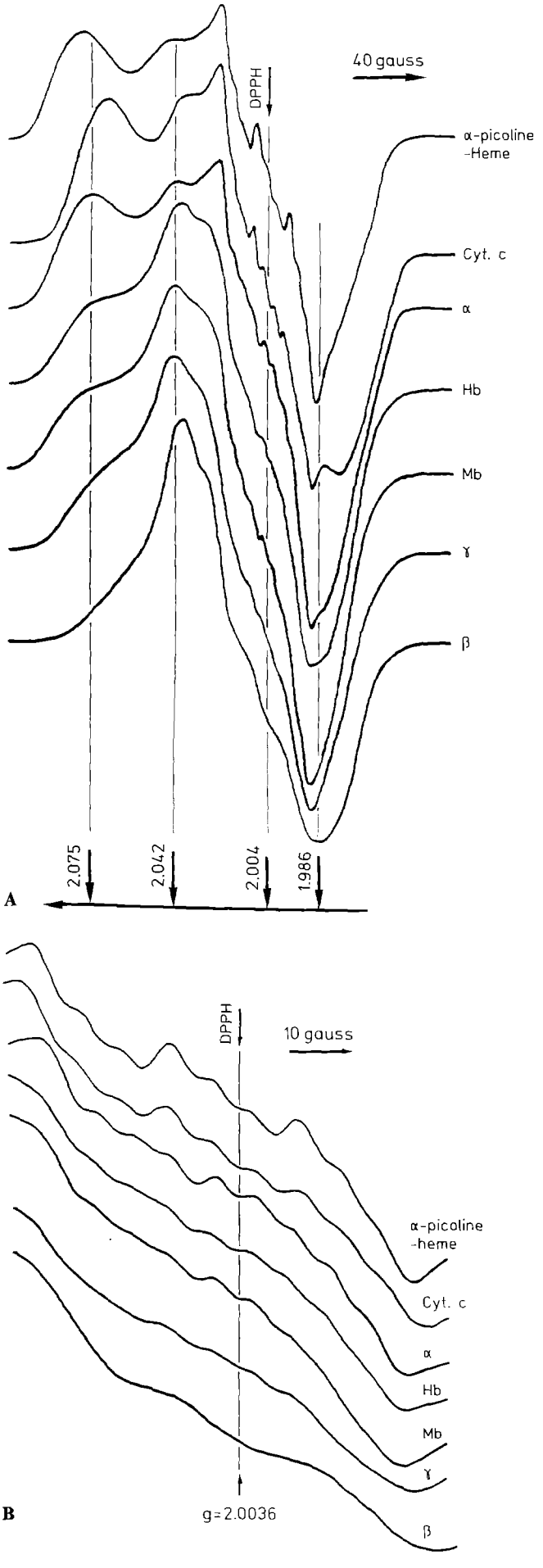
Table 1. Summary of characteristics of EPR spectra of nitrosyl-hemoproteins

Proteins and complexes	Temperature (K)	Type	g-values $g_x; g_z; g_y$	SHF (number)	A-values (gauss)	References
Hemoglobin (Hb)	77	C	2.060; 2.010; 2.005	9	26.8; 6.4	Kon (1968); Yonetani et al. (1972)
Heme + quinoline	77	A	2.100; 2.052; 2.008	3	16.0	Kon and Kataoka (1969)
Heme + acetylpyridine	77	B	2.081; 2.017; 2.009	3	16.5	Kon and Kataoka (1969)
Heme + pyridine	77	C	2.030; 2.007; 2.005	9	21.4; 6.5	Kon and Kataoka (1969)
Heme + imidazole/water	77	D	2.069; – ; 2.008	3	16.6	Kon and Kataoka (1969)
Hb; single crystals	77	–	2.082; 2.025; 1.991	–		Chien (1969 b)
Hb + IHP or pH < 6	77	B	– – –	3	16.5	Rein et al. (1972)
Hb + DPG or pH < 6	77	B	2.064; – ; 2.005	3	15	Trittelvitz et al. (1972)
alpha subunits	77	C	2.075; 2.004; 1.986	9	19; 6.5	Henry and Banerjee (1973)
beta subunits	77	C?	2.042; 2.004; 1.986	>3	22	Henry and Banerjee (1973)
Hb hybrids ("oxy")	77	C	2.075; 2.004; 1.988	9	19; 6.5	Henry and Banerjee (1973)
Hb hybrids ("deoxy")	77	B	2.075; 2.009; 1.988	>3	16	Henry and Banerjee (1973)
Mb; single crystals	77	C	2.072; 2.066; 1.985	9	24.5; 6.4	Dickinson and Chien (1971)
Myoglobin	77	C	2.080; 2.006; 1.980	9	30; 6.8	Yonetani et al. (1972)
Cytochrome c	77	C	2.078; 2.003; 2.003	9	24; 6.8	Kon (1969); Kon and Kataoka (1969)
Cytochrome c'	77	B	2.10 ; 2.03 ; 2.01	3	16	Yoshimura et al. (1988)
Catalase	77	B	2.050; 2.004; 1.97	3	21	Yonetani et al. (1972)
Catalase	93	B	– – –	3	–	Craven et al. (1979)
Chloroperoxidase	100	B	2.082; 2.004; 1.975	3	20	Chiang et al. (1975)
Myeloperoxidase	30	C	2.074; 2.001; 1.953	9	23.4; 6.9	Bolscher and Wever (1984)
Eosinophil Peroxidase	30	C	2.076; 2.002; 1.957	9	23.2; 7.3	Bolscher and Wever (1984)
Lactoperoxidase	30	C	2.087; 2.004; 1.962	9	20.9; 7.3	Bolscher and Wever (1984)
Lactoperoxidase	77	B	2.070; 2.004; 1.958	3	16	Yonetani et al. (1972)
Lactoperoxidase	30	B	2.09 ; 2.01 ; 1.97	3	17.5	Sievers et al. (1984)
Turnip P1, P2, P3, P7	77	C	2.072; 2.004; 1.960	9	21; 6.5	Henry and Mazza (1974)
Cyt. c Peroxidase	77	C	2.080; 2.004; 1.960	9	21; 6.4	Yonetani et al. (1972)
HorseRadish Peroxidase	77	C	2.080; 2.004; 1.955	9	20.5; 6.5	Yonetani et al. (1972)
Try-2,3-DiOxygenase	77	B	2.094; 2.009; 1.988	3	16.5	Henry et al. (1976)
TDO + L-Tryptophan	77	C	2.088; 2.004; 1.982	9	20; 7.0	Henry et al. (1976)
Cyt. P-450 CAMPHOR	77	C?	2.073; 2.009; 1.976	3	19.6	O'Keeffe et al. (1978)
Cyt. P-420 CAMPHOR	77	A	2.072; 2.015; 2.015	3	16	O'Keeffe et al. (1978)
Cyt. P-420	93	A	– – –	3	–	Craven et al. (1979)
Cyt. cd_1 (Nitrite-red)	77	C	2.060; 2.003; 1.960	9	21.4; 6.5	Bessières and Henry (1980)
Cyt. c Oxidase heme a_3	80	C	2.09 ; 2.005; 2.00	9	21.1; 6.8	Blokzijl-Homan and Van Gelder (1971)
CcO heme a_3	77	C	2.085; 2.004; 1.97	9	22; 7.3	Yonetani et al. (1972)
CcO heme a_3	77	C	2.092; 2.006; 1.980	9	21.4; 6.9	LoBrutto et al. (1983)
Cancer tissue	90	A	2.07 ; 2.03 ; 1.93	3	–	Vanin et al. (1970)
Cured meat	93	A	2.07 ; 2.03 ; 2.01	3	16.5	Bonnett et al. (1980)
CcO heme a_3-Cua	7	–	"$g=4$"; "$g=2$"	–	–	Stevens et al. (1979)
CcO	18	–	"$g=4$"; "$g=2$"	–	–	Boelens et al. (1982)

to hemes of type a (heme a_3 of cytochrome c oxidase of mammalian mitochondria or of *E. coli*), b (catalase, peroxidases, tryptophane-2,3-dioxygenase from *Pseudomonas fluorescens,* cytochrome P-450, etc) or d_1 (cytochrome oxidase d from *E. coli,* cyt. cd_1 (nitrite reductase) from *Pseudomonas aeruginosa*) (Table 1). It also binds to cytochrome c (Kon 1969; Kon and Kataoka 1969) by displacement of the methionyl residue and to cytochrome c' (Yoshimura et al. 1987, 1988). In these experiments NO was used as a probe of the heme site conformational changes induced by substrate binding (Henry and Mazza 1974; Henry et al. 1976; Bessières and Henry 1980) (Figs. 3 and 4). In many instances a nine SHF structure was found, indicating the binding of the nitrosyl-heme to a protein proximal N-base. In other cases, the three line structure due to NO was the only one observed (Table 1). As we have already mentioned in the case of hemoglobin, it would be of great interest to know whether in cases such as catalase, cytochrome c' and chloroperoxidase, the nitrosyl-heme complex is bound to the protein or is only imbedded in a protein fold, and to have a measure of the binding equilibrium constant. The same would be true in the case of cytochrome P-450 where the proximal proteic ligand is a mercaptide, probably cysteinyl residue, and NO displaces a weakly bound *trans* imidazole residue (Chevion et al. 1977). The P-450-NO complex transforms easily to the P-420-NO form of type A (O'Keeffe et al. 1979; Tsubaki et al. 1987).

4.3. Nitrosyl iron-sulfur proteins and other non-heme, non-Fe–S iron proteins

Nitric oxide and nitrite are known inhibitors of N_2 fixation in *Clostridium pasteurianum* and in *Rhizobium* bacteroides in soybean (Lockshin and Burris 1965; Trinchant



and Rigaud 1980), through binding to the Fe-S cluster of nitrogenase (Meyer 1981). Nitrosyl-iron-sulfur complex formation was, in fact, demonstrated by EPR for *Clostridium botulinum* (Reddy et al. 1983) and *Rhodopseudomonas sphaeroides* (Michalski and Nicholas 1987). The spectra are characterized by two g-values at 2.035 and 2.01, at 77 K (Table 2).

Similar spectra were earlier described by Salerno et al. (1976) for succinate dehydrogenase, a soluble Fe-S containing protein purified from complex II of beef heart mitochondria and also even earlier for Fe^{2+}–NO complexes with cysteine and aldolase (Vanin 1967; Woolum et al. 1968; Vanin et al. 1977) (Table 2). Fe^{2+}–NO complexes with imidazole, polyhistidine and bovine serum albumin are somewhat different, as they present a rhombic symmetry characterized by three g-values: e.g. 2.045, 2.033 and 2.013 for the Fe–NO-imidazole complex (Vanin 1967; Woolum et al. 1968; Woolum and Commoner 1970). Finally, similar spectra were detected in yeast cells (Vanin and Nalbandyan 1965) and in slices of mice or rat liver or tumors (Commoner et al. 1970; Woolum and Commoner 1970; Vanin et al. 1977, 1978) (Table 2).

The EPR characteristics just described are totally different from those found for other non-heme and non-Fe–S enzymes containing iron that could be complexed by nitric oxide, such as lipoxygenase, extradiol dioxygenases, etc (Table 2).

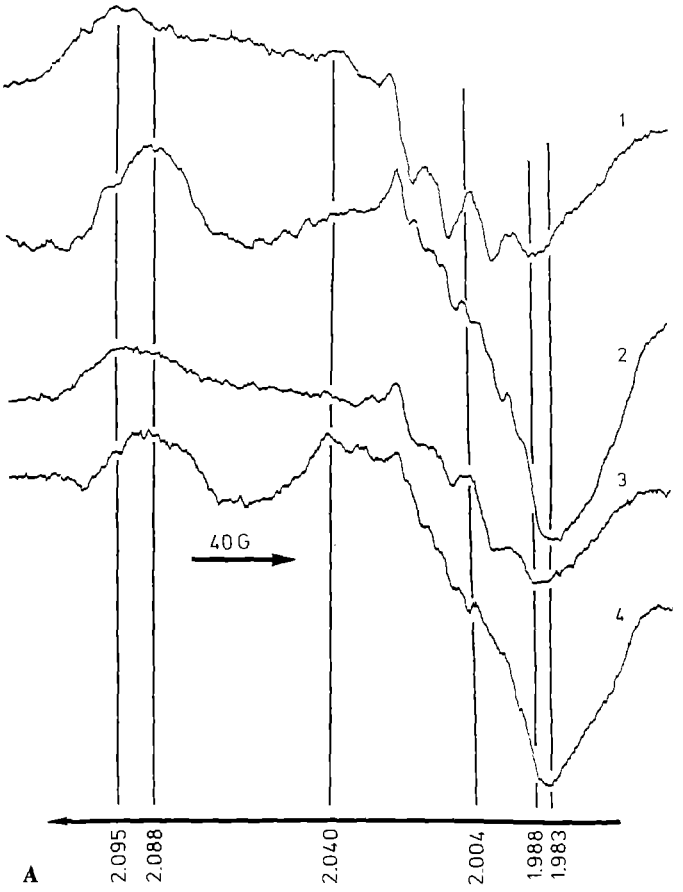
4.4. Nitrosyl multi-copper proteins and nitrosyl-hemerythrin

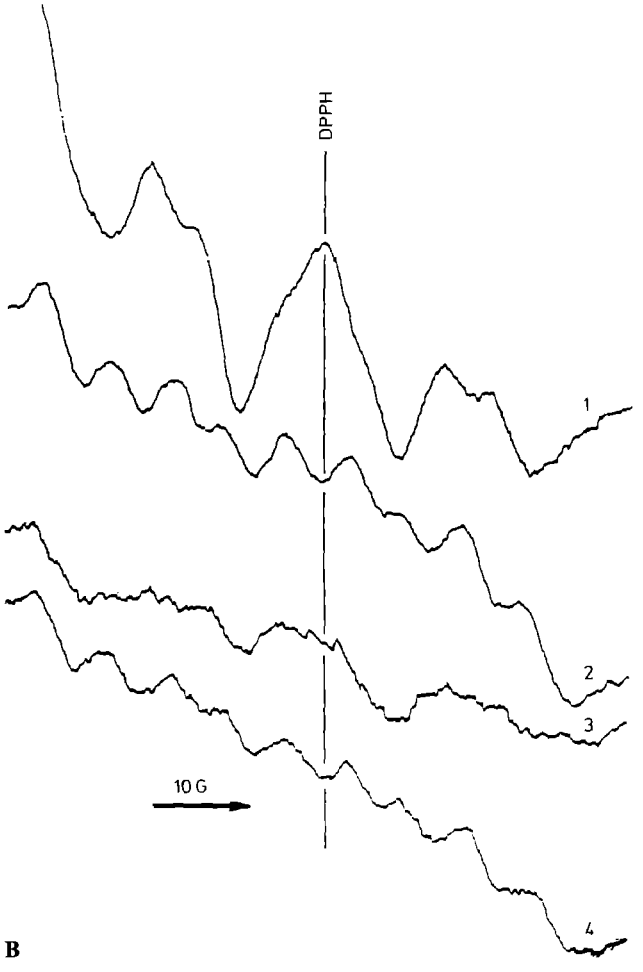
Nitric oxide binds to multi-copper proteins at the type 3 site composed of an EPR silent antiferromagnetically coupled copper pair (Solomon 1981), which is the oxygen binding site. Upon NO binding the two copper atoms are uncoupled and the EPR signals of Cu^{2+} complexes in the $g=2$ region, with often additional "forbidden" $\Delta M=2$ signals in the $g=4$ region, are detected. Such are the cases for tyrosinase and hemocyanin (Schoot-Uiterkamp and Mason 1973; Schoot-Uiterkamp et al. 1974; Verplaetse et al. 1979) and for the serum multi-copper oxidase, ceruloplasmin (Van Leeuwen and Van Gelder 1978). In the case of ascorbate oxidase (Van Leeuwen et al. 1975) and laccases from *Rhus vernicifera* and *Polyporus versicolor* (Rotilio et al. 1975; Martin et al. 1981) the type 1 copper

Fig. 2. **A** Frozen solution X-band EPR spectra of various nitrosyl-hemoproteins, human adult and fetal hemoglobin and subunits at 77 K. Heme concentration, 0.5 to 1 mM. Experimental settings: microwave frequency, 9.10 GHz; field modulation frequency, 100 kHz; field modulation amplitude, 1 G; microwave power, 10 mW. The stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used as a field marker. **B** Same as **A** with a 5-fold expanded field scan around $g=2$, showing the superhyperfine structures. Taken from Henry and Banerjee (1973) J Mol Biol 73:469–482. *Comments:* The comparison of the spectra of Hb, its subunits and Mb, which are structurally very similar hemoproteins, shows that the symmetry of the (F8) *His*-heme-NO paramagnetic centre varies greatly, being rhombic in αNO and nearly axial in βNO. The Hb spectrum is simply the sum of α and β contributions. Similarly, the nine SHF lines are easily detectable in α and totally blurred in β

Table 2. Summary of EPR spectral features of nitrosyl non-heme iron and copper proteins

Proteins and iron complexes	Organism	Temperature	g-values	References
Cysteine + Fe^{2+}		77 K	– ; 2.038; 2.014	Vanin (1967)
Cysteine + Fe^{2+}		−25 °C	– ; 2.036; 2.013	Woolum et al. (1968)
Aldolase + Fe^{2+}		−25 °C	– ; 2.036; 2.011	Woolum et al. (1968); Woolum and Commoner (1970)
BSA + Fe^{2+}		−25 °C	2.047; 2.038; 2.014	Woolum et al. (1968); Woolum and Commoner (1970)
Imidazole + Fe^{2+}		−25 °C	2.045; 2.033; 2.013	Woolum et al. (1968)
Polyhistidine + Fe^{2+}		−25 °C	2.055; 2.039; 2.014	Woolum et al. (1968)
	Yeast	77 K	– ; 2.03 ; 2.002	Vanin and Nalbandyan (1965)
	Rat liver	15 °C	– ; 2.035; 2.005	Commoner et al. (1970)
	Rat liver tumors	15 °C	– ; 2.035; 2.005	Woolum and Commoner (1970)
Rhodopsin		77 K	– ; 2.037; 2.012	Vanin et al. (1977)
Cysteine + Fe^{2+}		77 K	– ; 2.037; 2.012	Vanin et al. (1977); Vanin et al. (1978)
	Mice liver	77 K	– ; 2.037; 2.012	Vanin et al. (1978)
Succinate dehydrogenase	Beef heart mitochondria	77 K	– ; 2.035; 2.01	Salerno et al. (1976)
Cysteine + Fe^{2+} + ascorbate		77 K	– ; 2.035; 2.01	Salerno et al. (1976)
Iron-sulfur cluster	Clostridium botulinum	77 and 6.5 K	– ; 2.035; 2.01	Reddy et al. (1983)
Nitrogenase	Rhodopseudomonas sphaeroides	77 K	– ; 2.035; 2.01	Michalski and Nicholas (1987)
Transferrine	Human serum	77 K	2.055; 2.035; 2.015	Drapier, Pellat and Henry (1991, submitted)
BSA + Fe^{2+}		77 K	2.046; 2.038; 2.013	Drapier, Pellat and Henry (1991, submitted)
Ferritine	Horse spleen	77 K	– ; 2.038; 2.011	Drapier, Pellat and Henry (1991, submitted)
Ferredoxin	Spirulina platensis	77 K	– ; 2.041; 2.015	Drapier, Pellat and Henry (1991, submitted)
Ferredoxin	Porphyra umbilicalis	77 K	– ; 2.036; 2.015	Drapier, Pellat and Henry (1991, submitted)
Cysteine + Fe^{2+}		77 K	– ; 2.041; 2.014	Drapier, Pellat and Henry (1991, submitted)
Lipoxygenase-1	Soybean	15 K	"$g=4$"; "$g=2$"	Galpin et al. (1978); Salerno and Siedow (1979)
Lipoxygenase-1	Soybean	5.4 K	"$g=4$"; "$g=2$"	Nelson (1987)
	Neurospora crassa mitochondria	8.5 K	4.11; 3.95; 2.00	Rich et al. (1978)
Protocatechuate 4,5-dioxygenase	Pseudomonas testeroni	6 K	"$g=4$"; "$g=2$"	Arciero et al. (1985); Arciero and Lipscomb (1986)
Catechol 2,3-dioxygenase	Pseudomonas putida	6 K	"$g=4$"; "$g=2$"	Arciero et al. (1985)
Iron quinone complex	Spinach Photosystem II	4 K	$g=4$; –	Petrouleas and Diner (1990)
Ascorbate oxidase	Cucurbita pepo medullosa	88 K	Type 2 Cu^{2+} signal	Van Leeuwen et al. (1975)
Laccase	Rhus vernicifera	113 K	Type 2 Cu^{2+} signal	Rotilio et al. (1975)
Laccase	Rhus vernicifera	40 K	Type 2 and 3 Cu^{2+} signals	Martin et al. (1981)
Laccase	Polyporus versicolor	40 K	Type 2 Cu^{2+} signal	Martin et al. (1981)
Ceruloplasmin	Human serum	93 and 15 K	$g=4$; $g=2$; Type 2 signal	Van Leeuwen and Van Gelder (1978)
Tyrosinase	Agaricus bispora	113 and 14 K	$g=4$; $g=2$; Type 2 signal	Schoot-Uiterkamp and Mason (1973)
Hemocyanin	Helix pomatia; Cancer magister	113 and 14 K	$g=4$; $g=2$; Type 2 signal	Schoot-Uiterkamp et al. (1974)
Hemocyanin	Helix pomatia	97 K	$g=4$; $g=2$; Type 2 signal	Verplaetse et al. (1979)
Hemerythrin	Phascolopsis gouldii	4.5 K	2.77; 1.84	Nocek et al. (1988)





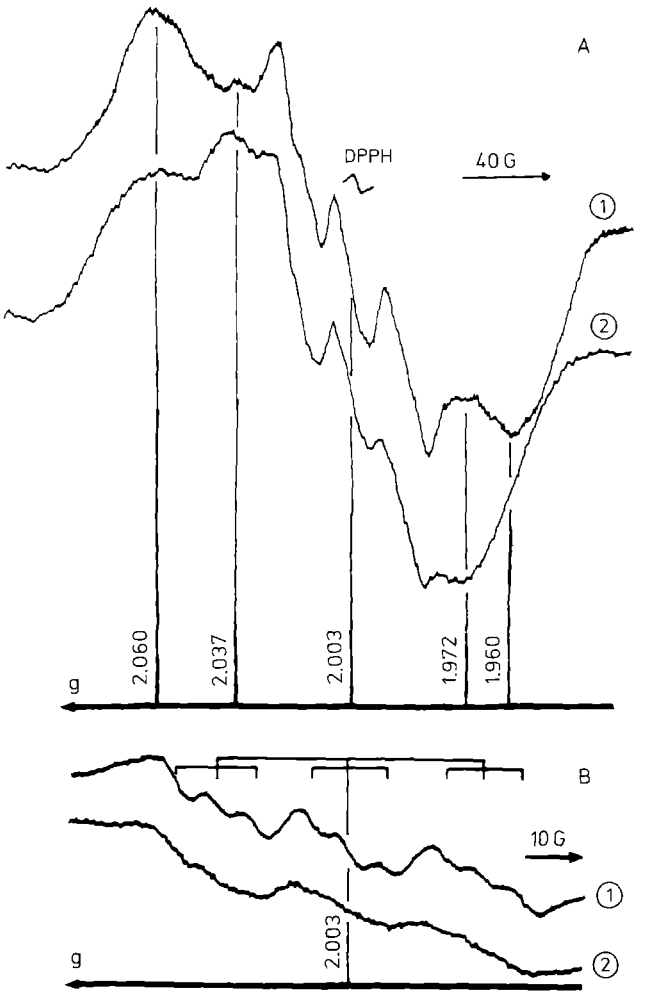


Fig. 4. **A** EPR spectra of *Pseudomonas aeruginosa* nitrite reductase (cyt. cd_1) at 77 K. *1:* Enzyme reduced by NADH and phenazine methosulfate (used as an electron mediator) and in the presence of NO (1 atmosphere). *2:* Enzyme having catalyzed the reduction of nitrite (77 m*M*) by NADH (2.5 m*M*) in the presence of phenazine methosulfate (77 μ*M*). Enzyme concentration, 100 μ*M*. Experimental settings as in Fig. 2. **B** Expanded field scan of **A**. Taken from Bessières and Henry (1980) CR Acad Sci 290:1309–1312. *Comments:* From the observed *g*-values, spectrum 2 can be interpreted as resulting from the heme *c*-NO moiety of the enzyme. In spectrum 2, there is a contribution of both heme *c*-NO and heme d_1-NO

Fig. 3. **A** EPR spectra of nitric oxide complexes of ferrous tryptophan-2,3-dioxygenase from *Pseudomonas fluorescens* in frozen solution at 77 K. *1:* TDO-NO alone; *2:* TDO-NO in the presence of L-tryptophan (2.5 m*M*); *3:* TDO-NO in the presence of α-methyltryptophan (2.5 m*M*), a regulatory effector of the enzyme; *4:* TDO-NO in the presence of α-methyltryptophan (2.5 m*M*) and of 5-hydroxytryptophan (1 m*M*), a competitive inhibitor with respect to L-tryptophan. Enzyme concentration, 35 μ*M*. Experimental settings: Microwave frequency, 9.16 GHz; modulation amplitude, 2 G; microwave power, 50 mW. **B** Expanded field scan of **A**. Taken from Henry et al. (1976) J Biol Chem 251:1578–1581. *Comments:* The SHF structure is essentially a three-line pattern, due to the ^{14}NO nitrogen ($A_z = 16.5$ G), in spectrum 1 and a nine-line pattern ($A_z = 20$ G and $A_z = 7$ G) in spectra 2 and 4. The addition of the substrate L-tryptophan or its competitive inhibitor, 5-hydroxytryptophan, corresponds to a strengthening of the bond between Fe^{2+} and the protein ^{14}N-atom and correlatively to a loosening of the Fe–NO bond

is reduced and the type 2 Cu^{2+} and one of the Cu^{2+} of the type 3 couple are detected without any magnetic interaction (Table 2).

In the case of the *Rhus* laccase, a catalytic cycle is set up upon addition of NO, in which NO_2^- and N_2O are formed, laccase acting as a NO-dismutase (Martin et al. 1981). In that respect, it is worthwhile to mention that mammalian dithionite-reduced or light-reduced cytochrome *c* oxidase can also reduce NO into N_2O (Brudvig et al. 1980; Rousseau et al. 1988). The same thing happens upon addition of NO to deoxyhemocyanin (Verplaetse et al. 1979). Cytochrome *c* oxidase, or hemocyanin, acts in that case, as does *Pseudomonas* cytochrome cd_1 (nitrite reductase). The biological significance of these catalytic reactions has yet to be ascertained.

Nitric oxide forms reversible adducts with another binuclear oxygen carrier protein, hemerythrin, at the (FeII–FeII) Deoxy and (FeII–FeIII) SemiMet oxidation levels (Nocek et al. 1988), here again decoupling the antiferromagnetic Fe–Fe site and giving rise to specific EPR species (Table 2).

5. Nitric oxide: biosynthesis and biological role

5.1. Macrophage synthesis of nitrate, nitrite and nitric oxide

Until the early 80's the presence of nitrate and nitrite in mammalian cells had been considered to be mostly of environmental origin. The first experiments demonstrating that nitrate biosynthesis was indeed occurring within mammalian cells, independently of gut flora participation, were carried out by Tannenbaum and co-workers (Green et al. 1981; Wagner et al. 1983), working on germ-free animals, which showed, in short, that nitrate excretion was greater than ingestion. The next fundamental advance was to show that nitrate synthesis was greatly enhanced by immunostimulation (Stuehr and Marletta 1985; and the reviews by Marletta 1988, 1989). This immunostimulation could be brought about in many ways, intraperitoneal injection of *E. coli* lipopolysaccharide (LPS) (Stuehr and Marletta 1985), injection of *Bacillus Calmette-Guérin* (BCG), treatment of macrophages by LPS, treatment by gamma-interferon (IFN-γ) and tumor necrosis factor (TNF) (Ding et al. 1988; Drapier et al. 1988).

The next stage was the demonstration in 1987 (Hibbs et al. 1987a; Iyengar et al. 1987) that nitrate and nitrite resulted from the oxidation of L-guanidino N atoms of L-arginine, in a newly discovered pathway leading to citrulline (for a review see Marletta 1989), independently of the urea cycle. Furthermore, Hibbs and co-workers (1987a) showed that the oxidation of L-arginine into nitrogen oxides is an effector mechanism causing target cell cytostasis as well as the reversible inhibition of the Krebs cycle enzyme, aconitase, by removal of the labile iron atom Fe_a (Beinert and Kennedy 1989) from the (4Fe–4S) cluster of aconitase (Drapier and Hibbs 1986). It leads also to the loss of activity of Complexes I and II of the electron transport chain in both macrophages and co-cultured tumor target cells (Drapier and Hibbs 1988; Hibbs et al. 1988; Stuehr and Nathan 1989). It was also found to be correlated with the inhibition of ribonucleotide reductase in adenocarcinoma cells (Lepoivre et al. 1990).

Finally, nitric oxide was proposed to be a precursor of nitrite and nitrate synthesis from L-arginine oxidation in activated macrophages (Marletta et al. 1988; Hibbs et al. 1988; Stuehr et al. 1989).

5.2. Nitric oxide as an endothelium-derived relaxing factor

In parallel to the discovery of the production of NO by stimulated macrophages, a simultaneous series of experiments led to the assignment of NO as an endothelium-derived relaxing factor (EDRF). From the earlier work of Furchgott and Zawadzki (1980), a factor released by endothelial cells was found to be necessary for the relaxation of vascular smooth muscle and inhibition of platelet aggregation and adhesion (Sneddon and Vane 1988; Radomski et al. 1990), induced by various substances such as acetylcholine, bradykinin or Ca^{2+} ionophore A23187. It was found to be labile (5 to 50 s half-life) and destroyed by O_2 and O_2^-. Moncada, Palmer and co-workers, and Ignarro and colleagues soon demonstrated that NO was indeed an EDRF (Ignarro et al. 1987; Palmer et al. 1987; Moncada et al. 1988) and was synthesized from L-arginine (Palmer et al. 1988a, b). This discovery led to the suggestion of a mechanism of action for long-known vasodilatory drugs such as amyl nitrite and nitroglycerin (see Sect. 6); NO is the endogenous nitrovasodilator (Moncada et al. 1988, 1989).

5.3. Activation of guanylate cyclase by nitric oxide and nitroso compounds

Guanylate cyclase (GC) (GTP pyrophosphate-lyase (cyclizing), E.C.4.6.1.2) has been known since 1977 to be activated by various agents, N_3^-, hydroxylamine NH_2OH, NO_2^-, nitroprusside, *N*-nitroso compounds and nitroglycerin. A common mechanism of activation was suspected, following the discovery that NO activates cytoplasmic GC and increases *c*GMP levels, but not *c*AMP's, in various tissue preparations (liver, kidney, spleen, lung, tracheal smooth muscle, small intestine muscle, heart, adrenal, cerebral cortex, cerebellum) (Arnold et al. 1977). GC can also be activated by S-nitrosothiols (Ignarro et al. 1980, 1981). Later, the involvement of the paramagnetic nitrosyl-heme complex in GC activation was demonstrated by two groups (Craven and DeRubertis 1978; Craven et al. 1979; Ignarro et al. 1982; Ohlstein et al. 1982). According to Ignarro et al. (1986), activation of GC by nitrosyl-hemoproteins such as HbNO or catalase-NO (Craven and DeRubertis 1978; Craven et al. 1979) involves NO-heme exchange.

6. Nitric oxide, a product in the metabolization of glyceryl trinitrate by *Phanerochaete chrysosporium*: detection by EPR of NO formation and binding to proteins

Glyceryl trinitrate (GTN), the explosive component of dynamite, has been used for a long time in the treatment

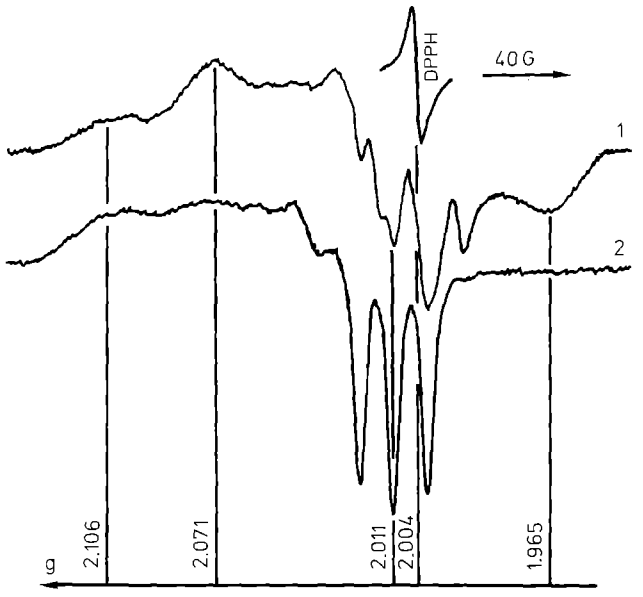


Fig. 5. EPR spectra at 77 K of cytochrome P-450-NO/P-420-NO from microsomal preparations of liver of dexamethazone-treated rats. *1:* After incubation under argon with nitrite (9 m*M*) and ascorbate (9 m*M*). *2:* After an hour incubation under argon with TNG (1.5 m*M*) and NADPH (15 m*M*). Cytochrome P-450 concentration, 48 μ*M*. Experimental settings: Microwave frequency, 9.18 GHz; microwave power, 10 mW; modulation amplitude, 4 G. (Servent, Ducrocq and Henry, unpublished results). *Comments:* Spectrum 2 is typical of a type A spectrum according to Kon and Kataoka, and can be attributed to a P-420-NO complex ($g_z = 2.01$; $A_z = 16$ G). In spectrum 1, the resonances at $g_x = 2.071$; $g_z = 2.004$ ($A_z = 20$ G) and $g_y = 1.965$ are assigned to a P-450-NO complex, with additional features in the $g = 2.106$ low field region and in the triplet centered at $g = 2.01$, which arise from an additional P-420-NO complex

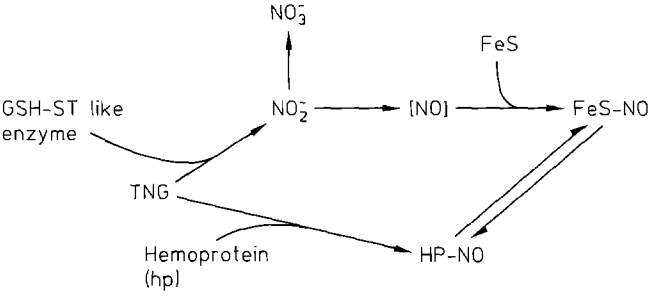
of cardiovascular diseases. Its pharmacological activity is directly related to the degradation of its nitrate functions. In mammals, a glutathione *S*-transferase could transfer a $-NO_2$ function to GSH to form $GS-NO_2$, which in turn would liberate NO_2^- and form GSSG. Another metabolic pathway involving cytochrome P-450 has been demonstrated in a microsomal hepatic fraction from phenobarbital-treated rats (Servent et al. 1989). It involves the formation of a P-450-NO complex identified by spectrophotometry. In experiments performed with dexamethazone-treated rat liver microsomes, we observed the formation of a P-420-NO complex after metabolic redution of TNG and of a mixture of P-450-NO and P-420-NO complexes after reduction of nitrite (Fig. 5).

In yeasts and fungi, Ducrocq et al. (1989, 1990) showed that GTN was selectively converted to 1,2-GDN and 1,3-GDN, 1-GMN and 2-GMN, with variable ratios depending on the strain used.

We have attempted to detect directly the formation of NO in the entire mycelium of *Phanerochaete chrysosporium*, grown aerobically, by EPR during the course of GTN metabolism, and compared it with the production of NO_2^- and NO_3^- (Servent, Ducrocq, Henry, Guissani and Lenfant, submitted 1990). The metabolism of nitrate and nitrite by resting cells was also determined as a blank.

We found firstly that the fungus in aerobiosis did not reduce nitrate to nitrite. On the contrary, nitrite can be partially oxidized to nitrate. We found, by EPR spectrometry that another part of nitrite was reduced to NO, forming a nitrosyl-Fe–S complex characterized by *g*-values at 2.042, 2.016 and 2.004, similar to those found for succinate dehydrogenase, nitrogenase and cysteine-Fe^{2+}-nitrosyl complexes (see Table 2).

The degradation of GTN did not produce nitrate, suggesting that no esterase-type activity was involved. GTN metabolism quickly produced a typical heme (Fe^{2+})–NO complex, with apparent *g*-values at 2.101, 2.078, 2.042 and 2.011 and a SHF coupling, related to the NO nitrogen, of 17 gauss. At longer times, a (Fe–S)–NO complex is also formed, analogous to that produced by the nitrite metabolism, that could arise from a glutathione *S*-transferase system. From these EPR data, and other kinetic data, a possible general enzymatic pathway can be proposed:



The EPR spectrum of the heme (Fe^{2+})–NO complex is of type A, according to Kon and Kataoka (1969), similar to that found for cytochrome P-420-NO or cytochrome *c'*-NO (see Table 1 and Sect. 4.2). It is likely that, in such cases, the strong bond between NO and the Fe atom can loosen or break the *trans* bond between the Fe atom and the protein, allowing the release of a heme-NO moiety. This mechanism may be relevant to the metabolism of GTN in mammalian cells. The formation of a heme-NO complex, loosely bound to a protein, could activate GC by heme-NO transfer, to trigger the vascular smooth muscle.

7. Nitric oxide derived from L-arginine in immunostimulated macrophages: detection by EPR of complexes between NO and non-heme iron proteins

As mentioned in Sect. 5.1, NO derived from L-arginine induces a loss of activity of aconitase, complexes I and II in activated macrophages as well as in co-cultured tumor target cells. To further characterize this phenomenon at a molecular level, we have determined, by EPR spectroscopy, whether coordination complexes between L-arginine-derived NO and iron-containing proteins could be detected in IFN-γ-activated macrophages. Release of nitrite in the culture medium and activity of aconitase were followed in parallel to EPR experiments.

In IFN-γ-activated peritoneal macrophages, as well as in the RAW 264.7 macrophage continuous cell line, we observed the appearance of a signal around $g = 2.04$ and 2.015, typical of nitrosyl-iron-sulfur complexes (see Fig. 6, Table 2 and Sect. 4.3) (Pellat et al. 1990 a, b).

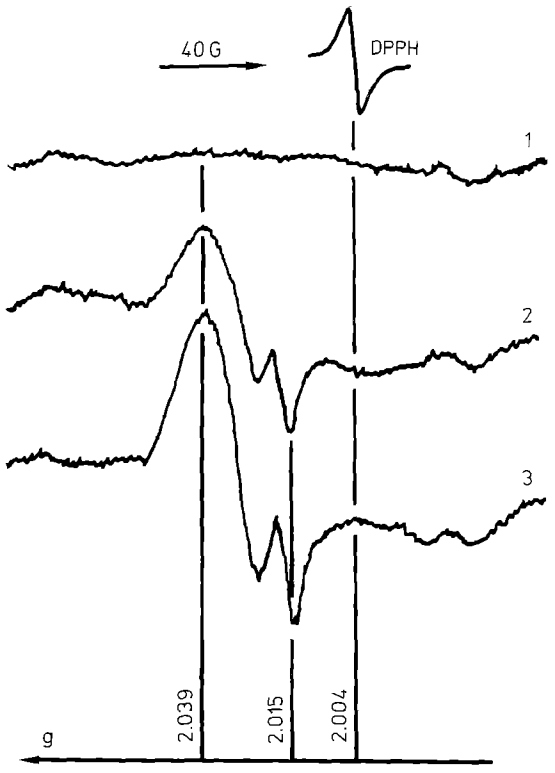


Fig. 6. EPR spectra at 77 K from digitonine-permeabilized macrophage continuous cell line RAW 264.7. Cells were incubated for 24 h in Dulbecco's modified essential medium alone (*1* and *3*), or added with 200 U/ml IFN-γ (*2*). *3:* An aliquot of digitonin-permeabilized macrophages was incubated with nitrite (5 m*M*) and ascorbate (5 m*M*) under helium for a further 30 min. Experimental settings: microwave frequency, 9.18 GHz; modulation amplitude, 10 G; microwave power, 10 mW. Taken partially from Pellat et al. (1990) Biochem Biophys Res Commun 166:119–125. *Comments:* the resonances arise from complexes between L-arginine-derived nitric oxide and non-heme iron proteins, identified as being, most probably, FeS proteins (Table 2) (Drapier, Pellat and Henry 1991, submitted)

No such signal occurred in the absence of IFN-γ, or when the culture medium did not contain L-arginine. The same was true when the medium, containing IFN-γ and L-arginine, also contained NMMA (N^G-monomethyl-L-arginine), a competitive inhibitor of NO synthesis from L-arginine. Another negative blank experiment was performed with P388D1 cells that cannot be induced by IFN or LPS to oxidize L-arginine into nitrogen oxides.

In all these case, no heme-NO signal was ever detected. As previously discussed (Sects. 4.3 and 5.1) for bacterial iron-sulfur enzymes, the possibility exists that NO can coordinate to such a cluster or remove reversibly the labile Fe_a atom, with resulting loss of enzyme activity. It is tempting to propose that NO can cross cell membranes to inhibit some mitochondrial Fe–S enzymes. Furthermore, it could explain, at a molecular level, the inhibition of Fe–S enzymes both in activated macrophages and target cells co-cultivated in close contact. Similar findings were obtained by Lancaster and Hibbs (1990) with BCG-activated macrophages.

Conclusion

We have attempted in this review to show the capacity of EPR spectroscopy to determine the presence and the molecular localization of paramagnetic species in complex whole cells or organs. The prerequisite for such demonstrations are sound chemical, biochemical and biophysical backgrounds, and the existence of a complete and properly interpreted spectroscopic dictionary.

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