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Nitrosyl hemoglobin: EPR components at low temperatures

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Abstract. The EPR spectrum of nitrosyl hemoglobin has been studied from 7.5 K to 104 K. It is composed of at least three components (A, B and C) which have a different dependence on temperature and power level. The A component decreases with increasing temperature. The B component disappears at around 30 K and is replaced by C. Relaxation of A follows the Orbach mechanism with an energy of 28 cm^{-1}. This behavior can be attributed to phonon induced changes in the orientation of NO with respect to the heme plane.

Key words: Nitrosyl hemoglobin – EPR components

Introduction

Nitrosyl hemoglobin and nitrosyl myoglobin (HbNO and MbNO) have been fairly thoroughly studied, mainly by Electron Paramagnetic Resonance (EPR) spectroscopy. This is due, in great part, to the fact that NO as a ligand of heme iron has a great deal of similarity in its electronic structure to the physiologically important oxygen. In both cases the iron atom is ferrous with spin zero. HbNO, however, is paramagnetic ($S=1/2$), while oxyhemoglobin (HbO_2) is diamagnetic.

Another reason for the interest in the EPR of HbNO (or MbNO) is that the observed complex spectra are sensitive to many factors, such as quaternary and tertiary structures of the proteins, concentration of NO, pH, degree of hydration, etc. (Sancier et al. 1962; Martin Neto et al. 1988; Sanches 1988). They also differ for NO bound to the α and β chains of Hb (Henry and Banerjee 1973; Schulman et al. 1975; Nagai et al. 1978; Taketa et al. 1978; Scholler et al. 1979; Louro et al. 1981).

More recently HbNO and MbNO have been used in kinetic studies of photodissociation (Cornelius et al. 1983; Jongeward et al. 1986). It was shown that EPR is a suitable technique for these studies (Nagai et al. 1978; Lo Brutto et al. 1984; Linhares et al. 1990). The saturation behavior over a wide temperature range is useful in providing a better understanding of the spectra and is relevant for defining the appropriate conditions for these EPR experiments.

In MbNO it has been shown that the spectra result from two and three conformations in thermal equilibrium in solution and in the crystal respectively (Morse and Chan 1980; Hori et al. 1981).

While EPR spectra of MbNO have been investigated over a wide temperature range (Morse and Chan 1980; Hori et al. 1981; Caracelli et al. 1988) the temperature and microwave power dependences of the EPR spectra of HbNO solutions have not yet been examined. In this work we study the behavior of the EPR signal of HbNO between 7.5 K and 104 K, as well as its dependence on microwave power. The saturation method appeared to be a convenient way to separate the overlapping spectra of HbNO which normally add to the complexity of the signal. This method also allows one to obtain the temperature dependence of the spin-lattice relaxation time T_1 of HbNO.

Experimental methods

Hb solutions were prepared by hemolysis of human blood and were stripped of ions by passage through a Sephadex G-25 (Sigma Co.) column. The solution was diluted to a heme concentration of 0.4 mM with 0.1 M phosphate buffer, pH 6.2. HbNO was obtained as described (Louro et al. 1981) by equilibration of the Hb samples with excess nitric oxide gas for at least 2 h before freezing.

EPR measurements were performed with an X-band spectrometer (Varian E-9) in the temperature range from 7.5 K to 104 K. The temperature of the sample was controlled by a helium flux cryostat (Helitran LTD-110) with an APD-E temperature controller (Air Products and Chemical). Temperatures were measured with an Au-Fe x

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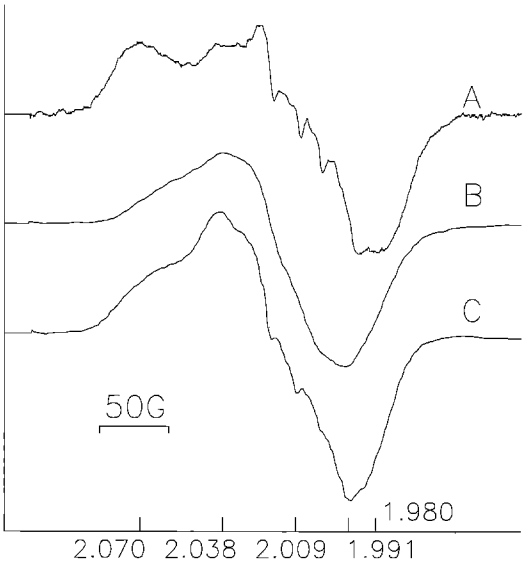

Fig. 1. EPR spectra of HbNO (0.4 mM, pH 6.2) A, B and C components. A at 7.5 K and 1.2×10^{-4} mW, B at 7.5 K and 225 mW and C at 104 K and 225 mW

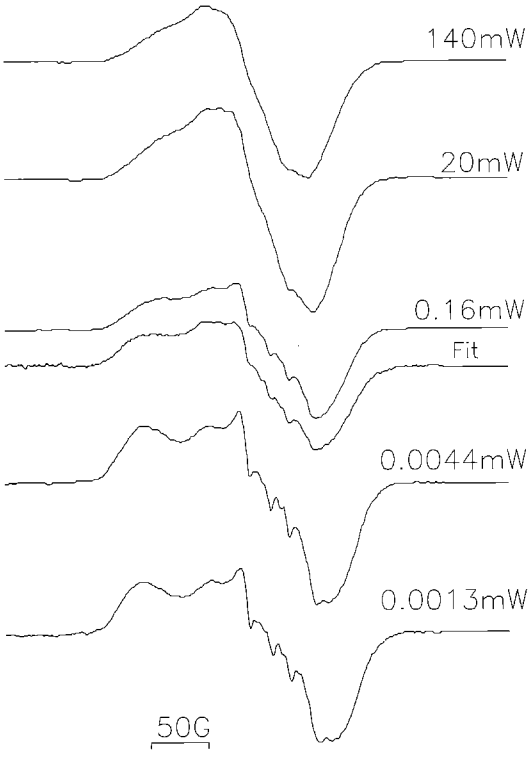

Fig. 2. Microwave power dependence of EPR spectra of HbNO (0.4 mM of heme, pH 6.2) at 7.5 K. Fit is the composite spectrum at 0.16 mW. From top to bottom, ratios of fractions of B/A are: 14.4, 4.8, 0.91, 0.17 and 0.1

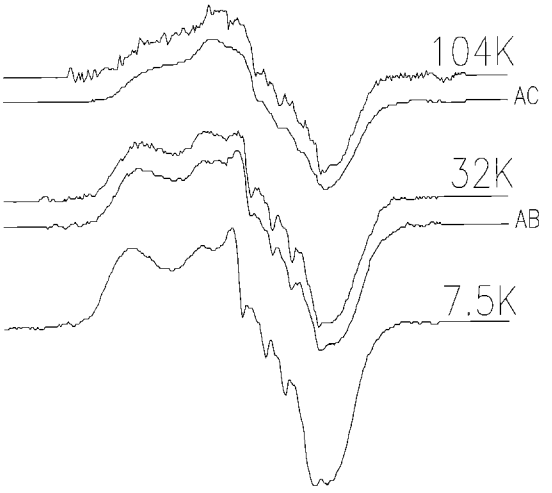

Fig. 3. Temperature dependence of EPR spectra of HbNO (0.4 mM of heme, pH 6.2) at 1.3×10^{-3} mW. AB is the composite spectrum at 32 K and AC at 104 K. From top to bottom, ratios of fractions are: C/A = 5, B/A = 0.25 and 0.1

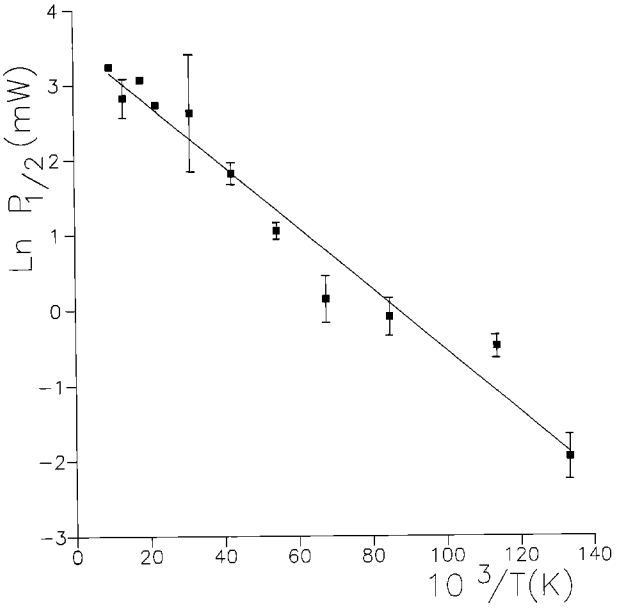

Fig. 4. Temperature dependence of $P_{1/2}$ (proportional to T_1^{-1}) of component A

Chromel thermocouple fixed on the tube's wall just above the sample.

Spectra were obtained for different microwave powers at each temperature. The g values and hyperfine splittings in Fig. 1 are the values measured from the extrema of derivatives. Spectrum A was taken at low temperature (7.5 K) and low microwave power (1.2×10^{-4} mW) and B at low temperature and high microwave power (225 mW). Spectrum C was taken at high temperature (104 K) and high microwave power. All spectra were reproduced by summing different fractions of A and B or A and C. The best composite spectra (Figs. 2 and 3) for each experimental one were chosen by inspection of different trial combinations.

The half saturation power, $P_{1/2}$, as well as estimated values of T_1 and T_2 (spin-lattice and spin-spin relaxation times, respectively) were obtained by the continuous wave saturation method as described previously (Yim et al. 1982 and Wajnberg et al. 1986).

The linear fittings of Figs. 4 and 5b were done using linear regression, minimizing the square root error.

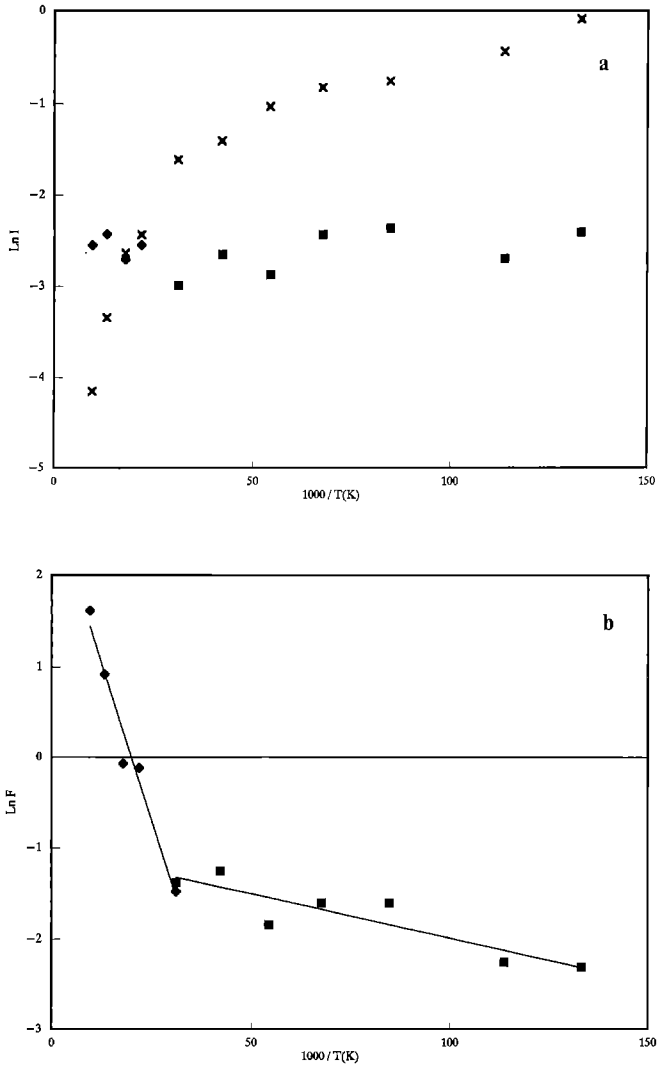

Fig. 5. **a** Temperature dependence of the intensity of components A (+), B (□) and C (∘) at 1.3×10^{-3} mW. **b** Temperature dependence of the ratios of intensity (F) of B (□) and C (∘) relative to A, at 1.3×10^{-3} mW

Results and discussion

Spectral analysis

The EPR signal was obtained as a function of temperature and microwave power.

At the lowest temperatures the shape of the signal is strongly dependent on microwave power. At low power one type of signal prevails (A signal). At higher power this signal saturates and decreases in amplitude. Another signal (B), very weak at low power, increases in amplitude with increasing power and dominates. Hence at 60 dB attenuation (1.2×10^{-4} mW) one obtains essentially a pure A spectrum and at 0 dB (225 mW) a pure B spectrum. A and B are the component spectra.

We observed in this work that with increasing temperature, the low microwave power spectrum undergoes a change in shape similar to that observed at low temperature and increasing microwave power. This is evident by comparison of the extreme spectra in Figs. 2 and 3. A similar temperature and microwave power evolution was also observed in HbNO crystals and it can not be attributed to the broadening of its components (Doetschman and Utterback 1981). To check if both spectral dependences are due to an equilibrium between conformations, we analyse each spectrum as a combination of two component spectra.

For temperatures up to 32 K all HbNO spectra were well reproduced by summing appropriate fractions of A and B. One spectral reconstruction at 7.5 K is shown as an example in Fig. 2, for 0.16 mW.

The A spectrum is asymmetric and shows a well resolved three line hyperfine structure as well as another weak structure centered at $g = 2.009$. They are indicative of interactions with the nitrogens of the nitroxide and of the proximal histidine (Henry and Banerjee 1973; Chevion et al. 1976/77). The hyperfine splittings are about 16 G and 7 G. Two other peaks are seen at $g = 2.070$ and $g = 1.985$. The B spectrum, centered at $g = 2.013$, is quite symmetric and shows no resolved hyperfine structure. Its linewidth is about 70 G.

Above 32 K it is not possible to reproduce any spectrum by summing A and B. The spectral changes become more apparent at temperatures closer to 104 K. For the reproduction of the spectra above 32 K one needs a combination of spectrum A with another spectrum C which replaces the low temperature B spectrum. The C spectrum shows only a slight indication of hyperfine structure (Fig. 1) at $g = 2.013$, with about 16 G splitting.

For temperatures higher than 32 K, even at high microwave power we do not observe any contribution of B to the spectra. Its disappearance cannot therefore be related to saturation. The temperature dependence of the spectra under non-saturating conditions and examples of reconstruction using A and B at 32 K and A and C at 104 K are shown in Fig. 3.

Since hemoglobin was saturated with NO the three spectra, A, B and C, belong to the R quaternary conformation. They resemble the spectra of nitrosyl isolated α or β chains, Hb (Henry and Banerjee 1973; Henry and Cassoly 1973; Scholler et al. 1979) or even Mb (Morse and Chan 1980) or erythrocruorin (Caracelli et al. 1988), whose details depend on pH, temperature and microwave power conditions.

The combination of different fractions of components A and B or A and C is sufficient to reproduce all the observed spectra. We have determined the contribution of the components A, B and C in the spectra at all microwave powers for each temperature.

In the following sections only the relative intensities of the components A, B and C are necessary in the analysis of the results. We have used these component spectra with normalized areas. The individual intensities were obtained from the fractions derived from this procedure, corrected for the spectrometer gain.

Relaxation

The relaxation behavior of the component spectra, A, B and C, was studied by the continuous saturation method.

This method yields experimentally the value of $P_{1/2}$ which is directly proportional to the relaxation rate T_1^{-1}. A plot of $P_{1/2}$ as a function of temperature (T) was obtained for the A spectrum and is shown in Fig. 4. The fit indicates that $T_1^{-1} = W \exp(-\Delta/kT)$, where $\Delta = 28\ cm^{-1}$, $W = 10^{5.1}$ and k is the Boltzman constant. This behavior is typical of the Orbach, two phonons relaxation mechanism with a characteristic energy Δ, observed previously for high spin ferric hemoglobins (Scholes et al. 1971) and for other proteins (Bertrand et al. 1982). Low spin ferric iron in general exhibits a T^n dependence (Stapleton 1986).

T_1 of the A component varies from 4.6×10^{-4} s at 10 K to 1.2×10^{-5} s at 100 K. Since B and C have high $P_{1/2}$ values, it is not possible to obtain a good fit of their saturation behavior. Nevertheless the values of T_1 are estimated to range from 6.3×10^{-5} s for the B spectrum at 7.5 K to 6.3×10^{-6} s for C at 104 K. Since the fittings are done with components of constant shape, the only variable being their relative intensities, we take T_2 (proportional to the linewidth) to be constant and temperature independent for all three spectra. We obtain $T_2 \cong 1.5 \times 10^{-9}$ s.

Our T_1 values are of the same magnitude as those measured in MbNO solutions between 4.2 K and 20 K (Muench and Stapleton 1985). They assumed a linear temperature dependence for T_1^{-1}, with no identification of the relaxation mechanism. We have checked that their results can be fitted by an exponential temperature dependence as well as a power one. Besides the differences between the two proteins, different temperature ranges are certainly responsible for the different T_1 temperature dependence. On the other hand, the saturation measurements (Doetschman and Utterback 1981) in HbNO crystals between 1.6 K and 4.2 K yield lower T_1 and higher T_2 estimates. It is however known that the linewidths in crystals and in solution are considerably different (Brill and Hampton 1979).

The observation of a difference of one order of magnitude between the relaxation rates of spectra A and B is consistent with the observed responses of the spectra to the microwave power at low temperature (Fig. 2). The two distinct EPR signals in MbNO solution also provide evidence of different saturation behavior and are fairly easily saturated at low temperature ($T < 20$ K) (Morse and Chan 1980).

Temperature dependence

From the saturation measurements of the A, B and C spectra it can be seen that at attenuations higher than 40 dB (one tenth of the smallest $P_{1/2}$) none of the three spectra is saturated. Figure 5a shows that at 50 dB (1.3×10^{-3} mW), the intensity of the A signal decreases with increasing temperature. The intensity of B is less sensitive to temperature below 30 K and vanishes at this temperature when C appears. The intensity of the C signal is almost constant up to 104 K. In consequence, at liquid nitrogen temperature, at which most spectra have been reported, we observe almost pure C spectrum, almost identical to other observed spectra (Henry and Banerjee 1973; Sanches 1988).

At 50 dB attenuation, the ratios of amplitudes (F) of B/A up to 30 K and C/A above 30 K are shown in Fig. 5b. The ratio of B/A below 30 K is almost constant. The absolute value of this ratio depends on microwave power and normalization conditions.

We believe that the changes observed at 30 K correspond to a change of the bond angle of the ligand (Fe–N–O). X-ray diffraction of HbNO (Deatherage and Moffat 1979) crystals gives about 150° for the Fe–N–O angle at room temperature while EPR measurements at 77 K give about 110° (Chien 1969). Changes of the EPR signal of MbNO solutions (Morse and Chan 1980) and crystals (Hori et al. 1981) demonstrate that the conformation of the bonding of NO is drastically altered upon freezing. Different conformation have also been deduced from infrared measurements of stretching bands in carboxy myoglobin (MbCO). Three different absorption peaks have been associated with three different CO angles with respect to the heme with differences of the order of 20 cm^{-1} in energy (Ansari et al. 1987).

Conclusions

The low temperature EPR studies of HbNO help in understanding this complex system. It became apparent that the conditions under which the EPR spectra are taken are of great importance. The level of the klystron power affecting the relaxation processes can completely change the features of the spectra, in contrast to the case with MbNO, where only temperature dependence has been observed. Care should be taken in the analysis of relaxation in that type of spectrum. Further experiments should be performed to elucidate the causes of the differences in the relaxation rates of the species.

The relaxation results provide evidence for a low lying energy level of 28 cm^{-1} for the A component. Since one does not expect such a small difference in energy between the ground and excited electronic states in the proposed energy level diagram (Doetschman 1980), we proposed that 28 cm^{-1} refers to a difference between two different geometries (conformations) of liganded heme, produced by different Fe–N–O angles.

The postulated reorientation of NO at 28 cm^{-1} can be induced by low frequency phonon modes of the exterior medium in the 10–100 cm^{-1} range (Jortner 1976). The decrease of the amplitude of the A spectrum above 32 K is consistent with this interpretation. Other effects however may also contribute to this decrease, as observed for MbCO (Ansari et al. 1987). It is also interesting to observe that the inelastic neutron scattering spectra of myoglobin show a maximum in the density of vibrational states at 25 cm^{-1} (Cusack and Dorster 1990). The amplitude of the A spectrum decreases above 30 K, without changing the linewidth, and the new spectrum C is of approximately the same area as B which is replaces. Since we do not find any new line, we are confronted with two possibilities: *1)* The decrease of the amplitude of A is due to the appearance of a paramagnetic center with a very fast relaxation, not observed by us, because of its linewidth or *2)* it is due to a change of the paramagnetic

A species to a diamagnetic one. In either case we believe that the conformation of NO changes to a new one.

The low temperature B species reorients to a C species at about 32 K. This reorientation changes the aspect of the spectrum, but both B and C remain paramagnetic with approximately the same EPR spectral intensity (Fig. 5a).

The observed effects do not allow for a straightforward relationship between A, B and C components and α and β chains. Although there is a strong similarity between A and α, and B and C and β, the spectra can also be associated with the different EPR species of MbNO (Morse and Chan 1980) which have only one polypeptidic chain or to the complex erythrocruorin (Caracelli et al. 1988). Hence the spectra are not necessarily related to the α and β chains. The EPR spectra of HbNO have a complex temperature and power dependence which may include some contribution of the spectra of α and β chains.

It became apparent, particularly in the optical work of the Illinois group (Ansari et al. 1987), that the ligands in the case of MbCO do not form a unique angle with the heme plane. The different groups of ligands exhibit different dissociation kinetics. We observe in HbNO, by a different technique, phenomena which we also attribute to ligand orientation. Future studies by EPR and other techniques should be able to throw more light on the identity of each spectral component observed in the present work. It remains of course to be seen whether these effects play any role under the physiological conditions.

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