Temperature Dependence of Q-Band Electron Paramagnetic Resonance Spectra of Nitrosyl Heme Proteins

Marco Flores, Eliane Wajnberg, and George Bemski
Centro Brasileiro de Pesquisas Físicas, 22290-180 Rio de Janeiro, Brazil

ABSTRACT The Q-band (35 GHz) electron paramagnetic resonance (EPR) spectra of nitrosyl hemoglobin (HbNO) and nitrosyl myoglobin (MbNO) were studied as a function of temperature between 19 K and 200 K. The spectra of both heme proteins show two classes of variations as a function of temperature. The first one has previously been associated with the existence of two paramagnetic species, one with rhombic and the other with axial symmetry. The second one manifests itself in changes in the *g*-factors and linewidths of each species. These changes are correlated with the conformational substates model and associate the variations of *g*-values with changes in the angle of the N(his)-Fe-N(NO) bond in the rhombic species and with changes in the distance between Fe and N of the proximal (F8) histidine in the axial species.

INTRODUCTION

Nitrosyl hemoglobin (HbNO) and nitrosyl myoglobin (MbNO) have been fairly thoroughly studied by X-band electron paramagnetic resonance (EPR) spectroscopy. This was due in great part to the fact that the paramagnetic heme iron-NO system displays a great deal of similarity in its electronic structure to that of the physiologically important heme iron-O_2, which is, however, diamagnetic. Today it has gained in importance, because it has been shown recently that NO as a ligand of heme protein can be physiologically important (Jia et al., 1996).

Another reason for the interest in the EPR of HbNO and MbNO is that the observed, complex spectra are sensitive to many factors, such as tertiary and quaternary structures of proteins, α- and β-chains of Hb, concentration of NO, pH, degree of hydration, temperature, microwave power, etc. (Henry and Banerjee, 1973; Perutz et al., 1976; Chevion et al., 1978; Mun et al., 1979; Morse and Chan, 1980; Hori et al., 1981; Neto et al., 1988; Wajnberg et al., 1992, 1996; Hüttermann et al., 1994). Some of these experiments point to the existence of two conformations: rhombic and axial.

Different conformations have also been proposed for carboxyl myoglobin (MbCO) based on spectroscopic measurements (Hong et al., 1990). Discovery of three different absorption peaks in the infrared spectra of MbCO have been associated with three different CO angles with respect to the heme plane, and constituted the first detailed proof of the conformational substates model proposed by Frauenfelder et al. (1991). These absorption peaks were also observed in HbCO (Potter et al., 1990). Theoretical ideas suggest that these substates are a general property of proteins (Honeycutt and Thirumalai, 1990).

Whereas X-band EPR spectra of HbNO and MbNO have been investigated over a wide temperature range, studies of the temperature variation of their Q-band spectra have been limited (Hüttermann et al., 1994). In the present work we study the behavior of the Q-band spectra of both heme proteins between 19 K and 200 K. The better resolution of the Q-band, as compared to the X-band, due to its higher frequency, together with computer simulation, allows us to separate the complicated and overlapping spectra into their components.

MATERIALS AND METHODS

Hb was prepared from fresh human blood by standard procedures (Louro et al., 1981), the pH 6.5 was fixed by using Sephadex G-25 columns and phosphate buffer, and HbNO in saturated conditions was prepared by deoxygenating the sample with N_2 flux, followed by injection of NO gas. Mb (horse heart; Sigma) was diluted in phosphate buffer (pH 6.5) and completely reduced with sodium dithionite and kept in anerobic conditions with N_2 flux. MbNO was prepared by injection of NO gas.

Q-band EPR measurements were performed with a Q-band spectrometer (ESP300E; Bruker) from 19 K to 200 K, using a helium flux cryostat (ER 4118 CF; Oxford) with a temperature controller (Oxford ITC4). Temperatures were measured with a chromel versus alumel thermocouple placed below the sample. The spectra were studied at two fixed power levels of 0.003 mW and 0.97 mW, with a modulation amplitude of 1 gauss.

At low temperatures and high power, a wide and asymmetrical background signal was observed, practically overwhelming the whole spectrum. This background is obtained experimentally at different temperatures and subtracted from the whole spectrum. The resultant spectra are fitted with a simulation program (QPOWA program) for powdered samples (Belford and Nilges, 1979). This program considers the following parameters: *g*-factors, linewidths, hyperfine interactions, *g* strain, and superhyperfine interactions.

The *g*-factors are obtained from the QPOWA program, because the observed spectra are a sum of two (rhombic and axial). After the spectra are normalized by a second integration, the program is used to simulate the components of each spectrum independently. Experimentally these components are not fully resolved (Fig. 1). The components are multiplied by factors chosen so as to have their sum give the best fit to the experimental data. The sum of these factors is 1, because the areas of the spectra are constant within the experimental error. These factors represent, then, the fractions of each component in the spectra.

Received for publication 5 May 1997 and in final form 2 September 1997.

Address reprint requests to Dr. George Bemski, Centro Brasileiro de Pesquisas Físicas, Rua Xavier Sigaud 150, 22290-180, Rio de Janeiro, Brazil. Tel.: 55-21-541-0337; Fax: 55-21-541-2047; E-mail: bemski@cat.cbpf.br.

0006-3495/97/12/3225/05 $2.00

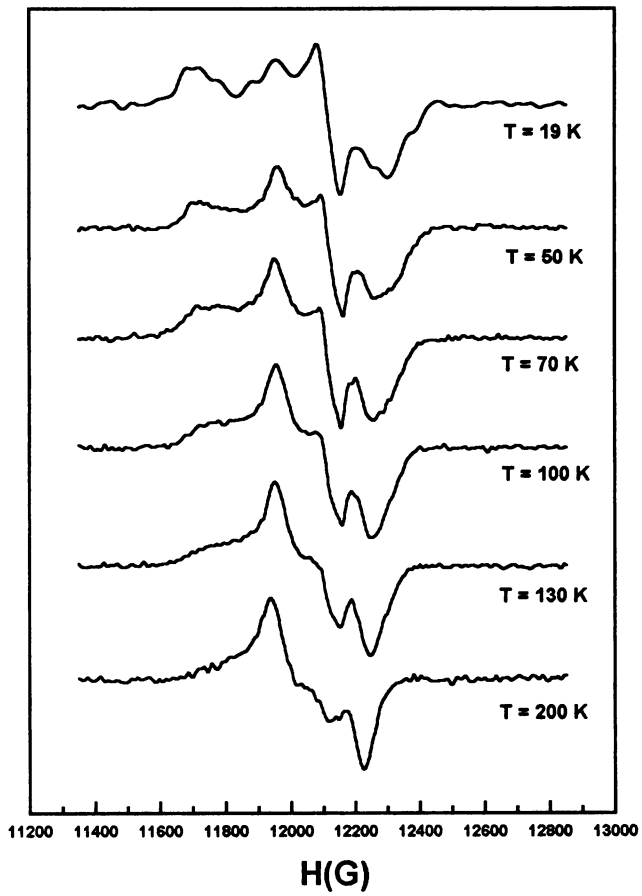

FIGURE 1 The Q-band EPR spectra of HbNO as a function of temperature at 0.97 mW.

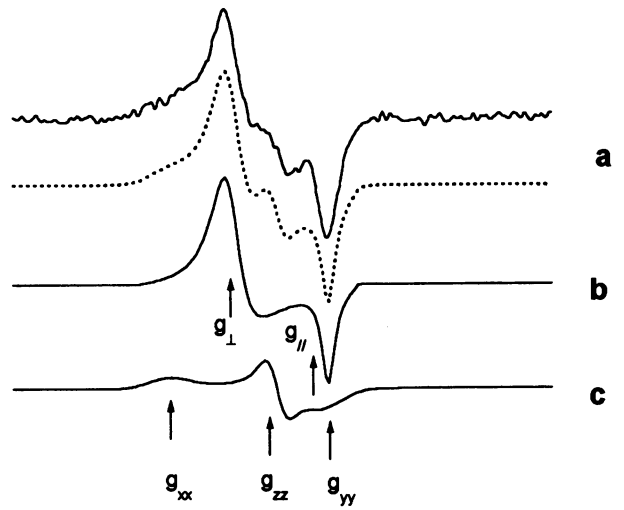

FIGURE 2 (*a*) Experimental (——) and fitted (- - -) spectra of HbNO at 200 K. (*b*) Axial component ($g_\perp$ = 2.032 and $g_\parallel$ = 1.987). (*c*) Rhombic component (g_{xx} = 2.062, g_{yy} = 1.989, g_{zz} = 2.010).

RESULTS

The spectra show five lines for both heme proteins. They can be associated with a sum of spectra due to two species of rhombic and axial symmetry (Hüttermann et al., 1994). Fig. 1 shows that the spectra vary significantly with temperature. The axial species predominates at high temperatures, and the rhombic species at low temperatures.

The spectra of each heme protein were fitted at eight temperatures. Fig. 2 shows a typical fit superimposed on the experimental spectra. g_{xx}, g_{yy}, and g_{zz} are g-factors of the rhombic component, and $g_\parallel$ and $g_\perp$ are g-factors of the axial component.

Good fits can only be achieved when g-factors and linewidths are temperature dependent, because there is an evident shift of the position of the absorption lines and variation in the linewidths (Fig. 3).

For both heme proteins, the variation in g_{xx} and g_{yy} of the rhombic component is significant, whereas g_{zz} is constant within experimental error. In HbNO and MbNO, g_{xx} decreases with increasing temperature from 2.076 to 2.062, and g_{yy} increases from 1.974 to 1.989 for HbNO, and from 1.982 to 1.989 for MbNO. The variations in $g_\perp$ and $g_\parallel$ of the axial component are also pronounced; in HbNO $g_\perp$ increases with increasing temperature from 2.028 to 2.032, and $g_\parallel$ decreases from 1.999 to 1.987, whereas in MbNO $g_\perp$ increases from 2.028 to 2.035 and $g_\parallel$ increases from 1.969 to 1.991.

We observe a large decrease in the linewidth in the parallel direction of the axial component of HbNO and MbNO above 70 K and 40 K, respectively, probably indicating an increase in the rate of rotation of NO.

The fractional abundance of the two species as a function of temperature was measured at low (0.003 mW) and high (0.97 mW) power levels to verify possible saturation effects. Because we did not observe such effects at 0.97 mW, we selected this power level to obtain better resolution.

DISCUSSION AND CONCLUSIONS

It was apparent for many years that the spectra of some nitrosyl adducts of Fe (II) protoporphyrins, as well as of some nitrosyl heme proteins, show complex EPR spectra, which in some cases can only be reproduced by adding varied fractions of two spectra. Hence the variation in the spectra with temperature is due to an equilibrium between two species. Morse and Chan (1980) showed it for sperm whale MbNO between 30 K (g_{xx} = 2.080, g_{yy} = 1.979, and g_{zz} = 1.998) and 180 K ($g_\perp$ = 2.041 and $g_\parallel$ = 1.983). These g-values are in reasonable agreement with those observed in this work. Hori et al. (1981) from their studies of single crystal of sperm whale MbNO conclude that the Fe-N-O bond angle is 153° at room temperature and 109° at 77 K.

Wajnberg et al. (1992) studied the X-band spectra of HbNO between 7.5 K and 104 K. They are composed of at least three components with different temperature and microwave power dependence. All spectra were reproduced by combining different fractions of two of the three components.

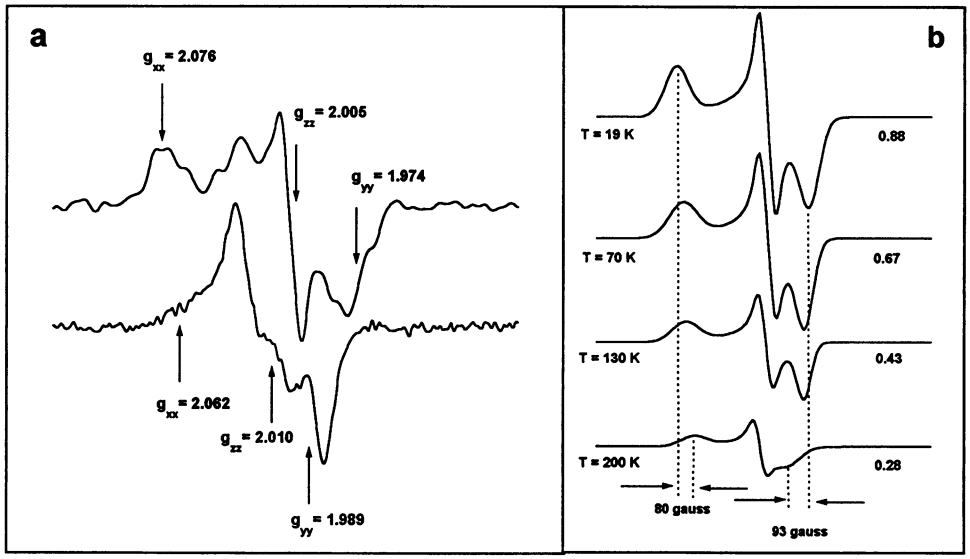

FIGURE 3 (*a*) Shift of the rhombic *g*-factors for HbNO with temperatures between 19 K (*top*) and 200 K (*bottom*). (*b*) Temperature dependence of the rhombic specie simulated for HbNO between 19 K and 200 K, showing the shift in gauss: 80 gauss for g_{xx} and 93 gauss for g_{yy}. The numbers on the right indicate the rhombic fractions.

Hüttermann et al. (1994) studied the X-band and Q-band spectra of nitrosyl-hemoglobin (HbNO), tetraphenyl porphyrin-imidazole (NO-TPP-Im), α-chain (α-NO), and β-chain (β-NO). The spectra clearly showed a superposition of a rhombic and an axial component in all of the samples, but variation in *g*-values with temperature was not considered by Hüttermann et al. (1994). This was probably due to insufficient resolution of X-band spectra and to the temperature range used (130–298 K) in the Q-band experiments. Indeed, in this temperature range we observed reasonable changes only for the g_{xx} and g_{yy} of the rhombic species of HbNO (g_{xx} from 2.069 to 2.062, g_{yy} from 1.978 to 1.989). The variation of the spectra with temperature was analyzed in terms of two different stereochemistries of the N(imidazole)-Fe-N(NO) bonds with respect to the porphyrin plane: one is an axial spectrum with the bond direction (and $g_{\|}$) parallel to the normal to the porphyrin plane; the other is one in which that direction is inclined to the normal to the porphyrin plane and gives rise to a rhombic spectrum. These were identified with the help of ENDOR (electron nuclear double resonance) experiments. The *R-T* transition in HbNO was reported to involve α-chain conformational changes only comprising a partial loss of the proximal histidine in the isolated subunits and in the tetrameric HbNO.

In the present work we find a similar temperature dependence of the Q-band spectra of HbNO and MbNO between 19 K and 200 K. We observe two types of spectral variations as a function of temperature. The first one is described by the equilibrium between axial and rhombic conformations. The second one is more subtle and manifests itself in temperature variations of the *g*-factors of each of these conformations, which points to the existence of more than a single rhombic and axial conformation as a function of temperature.

In the rhombic component this variation can be seen in the difference between g_{xx} and g_{yy} as a function of temperature. This variation is larger in HbNO than in MbNO (Fig. 4). The results suggest the existence of intermediate states with g_{xx}-g_{yy} constant for some ranges of temperature. This result can be correlated with different NO angles with respect to the heme plane (Hong et al., 1990). The decrease in g_{xx}-g_{yy} as temperature increases points to the decrease in rhombicity.

The axial component variation is seen in the changes in $g_{\|}$ with temperature and can be correlated with changes in the distance between Fe and N of the proximal histidine. Such changes have been reported by Cupane et al. (1993) for deoxy-Hb and deoxy-Mb.

Assuming the existence of axial (*A*) and rhombic (*R*) species only, it is possible to obtain the fraction of each species as a function of temperature. Using the two-state

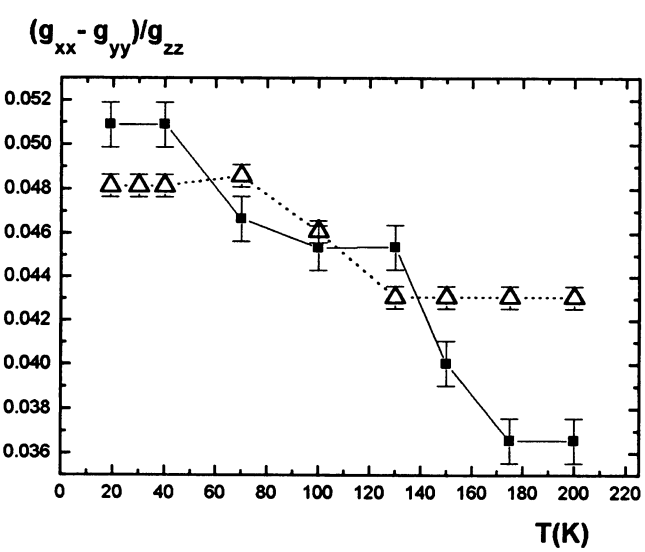

FIGURE 4 The difference between g_{xx} and g_{yy} as a function of temperature for HbNO (■) and MbNO (Δ).

model (Caracelli et al., 1988) and assigning free energies ΔG_A and ΔG_R to them, one obtains

$$\frac{A}{R} = \frac{\exp(-\Delta G_A/KT)}{\exp(-\Delta G_R/KT)} = \exp(-\Delta G/KT) \qquad (1)$$

where $\Delta G = \Delta G_A - \Delta G_R$ is the free energy difference between the two states. Considering that $\Delta G = \Delta H - T\Delta S$ and assuming that ΔH and ΔS are temperature independent, we plot $\ln(A/R)$, obtained directly from the fitting procedures, as a function of the inverse of temperature (Fig. 5). The deviation of the linear behavior points to the existence of more than two conformational states, in agreement with the observed g-factor variations.

Our results are in agreement with the model proposed by Ansari et al. (1994), which incorporates the results of dissociation kinetics in MbCO and assumes the existence of at least two deoxyheme infrared spectra and therefore two protein conformations. These two conformations can be correlated with the rhombic and axial species observed here, because experiments show that these conformations are independent of the ligand. The temperature variation of each species agrees with the conformational substates model (Frauenfelder et al., 1991). MbNO and HbNO differ only slightly in the variations in the rhombicity and in the ratio of the conformations with temperature (Figs. 5 and 6).

Shiga et al. (1969) studied the EPR spectrum of hemoglobin α_{NO} and β_{NO} and found that the HbNO spectrum is a sum of the α_{NO} and β_{NO} spectra. The near-identity between HbNO and MbNO results proves that their interpretation cannot depend on the presence of α- and β-chains in HbNO.

Another observation is the difference between the linewidths of the axial components in HbNO and MbNO (larger in HbNO). This result is in line with the findings of Bizarri and Cannistraro (1993), who studied by EPR high-

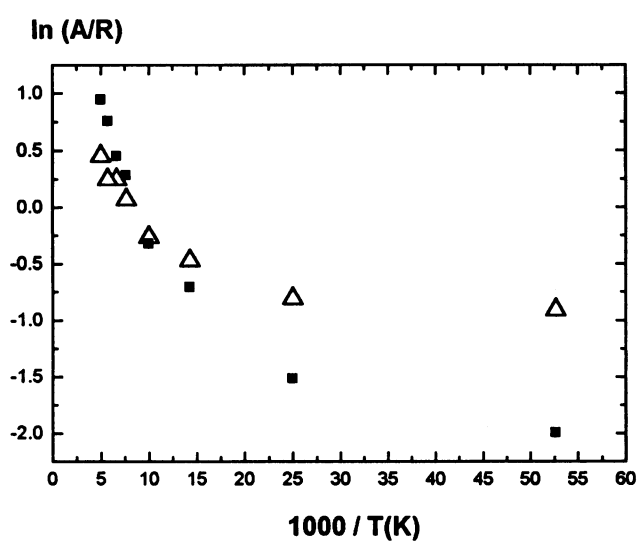

FIGURE 5 The relative fraction, A/R (axial to rhombic), as a function of inverse temperature for HbNO (■) and MbNO (Δ).

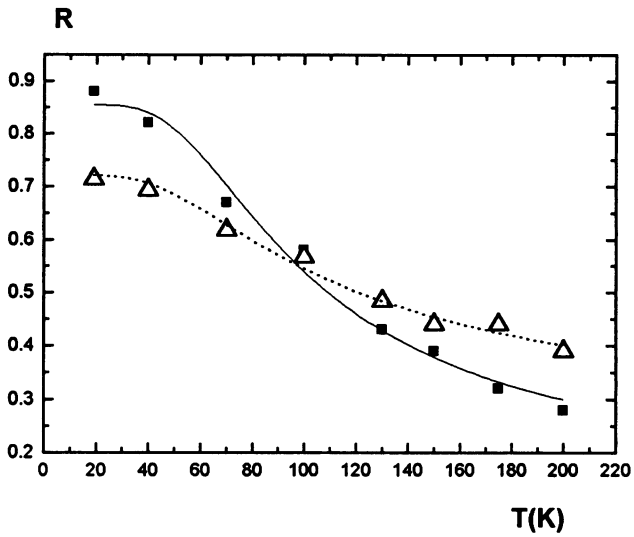

FIGURE 6 The rhombic fraction (R) as a function of temperature for HbNO (■) and MbNO (Δ).

spin FeIII myoglobin in solution. Their spectra were analyzed in terms of a distribution of the two crystal field parameters, due to a distribution in the displacement of heme iron along the normal to the heme plane. Our results may be due to the fact that HbNO has four chains and hence a larger distribution of g-values than MbNO, which has only one chain. This leads to a larger linewidth and is compatible with the larger flexibility of HbNO, as seen in the rhombicity results (Fig. 6).

In conclusion, the present experiment can be interpreted in the framework of the conformational model (Lim et al., 1995). The principal aspect of this model is the existence of various conformational species in thermodynamic equilibrium (Austin et al., 1975). Our experiments show that this is a general property of heme proteins, regardless of their quaternary structure (four chains in Hb and one in Mb). The study of the Q-band EPR components contributes to an understanding of the dynamic changes of heme proteins, which are certainly important in their biological functions.

The authors are grateful to Odivaldo Cambraia, Delson Schramn, and Elena Mavropoulos for technical assistance; the Instituto de Hematologia (Rio de Janeiro, Brazil) for blood samples; and CNPq and CAPES for partial support of this work.

REFERENCES

Ansari, A., C. M. Jones, E. R. Henry, J. Hofrichter, and W. A. Eaton. 1994. Conformational relaxation and ligand binding in myoglobin. *Biochemistry.* 33:5128–5145.

Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* 14:5355–5373.

Belford, R. L., and M. J. Nilges. 1979. Computer simulation of powder spectra. EPR Symposium, 21st Rocky Mountain Conference, Denver, CO.

Bizarri, A. R., and S. Cannistraro. 1993. Solvent modulation of the structural hetrogeneity in FeIII myoglobin samples: a low temperature EPR investigation. *Eur. Biophys. J.* 22:259–267.

Caracelli, I., N. C. Meirelles, M. Tabak, O. B. Filho, and O. R. Nascimento. 1988. An ESR study of nitrosyl *Aplysia brasiliana* myoglobin and nitrosyl annelidae *Glossoscolex paulistus* erythrocruorin. *Biochim. Biophys. Acta.* 955:315–320.

Chevion, M., J. M. Salhany, J. Peisach, W. E. Blumberg, and S. Simon. 1978. Analogous effect of protons and inositol hexaphosphate on the alteration of structure of nitrosyl fetal human hemoglobin. *Biochemistry.* 17:1745–1750.

Cupane, A., M. Leone, and E. Vitrano. 1993. Protein dynamics: conformational disorder, vibrational coupling and anharmonicity in deoxyhemoglobin and myoglobin. *Eur. Biophys. J.* 21:385–391.

Frauenfelder, H., S. G. Sligar, and P. G. Wolynes. 1991. The energy landscapes and motions of proteins. *Science.* 254:1598–1603.

Henry, Y., and R. Banerjee. 1973. Electron paramagnetic studies of nitric oxide haemoglobin derivatives: isolated subunits and nitric oxide hybrids. *J. Mol. Biol.* 73:469–482.

Honeycutt, J. D., and D. Thirumalai. 1990. Metastability of the folded states of globular proteins. *Proc. Natl. Acad. Sci. USA.* 87:3526–3529.

Hong, M. K., D. Braunstein, B. R. Cowen, H. Frauenfelder, I. E. T. Iben, J. R. Mourant, P. Ormos, R. Scholl, A. Schulte, P. J. Steinbach, A. H. Xie, and R. D. Young. 1990. Conformational substates and motions in myoglobin. *Biophys. J.* 58:429–436.

Hori, H., M. Ikeda-Saito, and T. Yonetani. 1981. Single crystal EPR of myoglobin nitroxide. *J. Biol. Chem.* 256:7849–7855.

Hüttermann, J., C. Burgard, and R. Kappl. 1994. Proton ENDOR from randomly oriented NO-ligated haemoglobin: approaching the structural basis for the R-T transition. *J. Chem. Soc. Faraday Trans.* 90: 3077–3087.

Jia, L., C. Bonaventura, J. Bonaventura, and J. S. Stamler. 1996. S-Nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature.* 380:221–226.

Lim, M., T. Jackson, and P. Anfinrud. 1995. Binding of CO to myoglobin from a heme pocket docking site to form nearly linear Fe-C-O. *Science.* 269:962–966.

Louro, J. R. W., P. C. Ribeiro, and G. Bemski. 1981. EPR spectral changes of nitrosyl hemes and their relation to the hemoglobin T-R transition. *Biochim. Biophys. Acta.* 670:56–63.

Morse, R. H., and S. I. Chan. 1980. Electron paramagnetic resonance studies of nitrosyl ferrous heme complexes. *J. Biol. Chem.* 255: 7876–7882.

Mun, S. K., J. C. Chang, and T. P. Das. 1979. Origin of observed changes in ^{14}N hyperfine interaction accompanying R $\rightarrow$ T transition in nitrosylhemoglobin. *Proc. Natl. Acad. Sci. USA.* 76:4842–4846.

Neto, L. M., O. R. Nascimento, M. Tabak, and I. Caracelli. 1988. The mechanism of reaction of nitrosyl with met- and oxymyoglobin: an ESR study. *Biochim. Biophys. Acta.* 956:189–196.

Perutz, M. A., J. V. Kilmartin, K. Nagai, A. Szabo, and S. R. Symon. 1976. Influence of globin structures on the state of the heme. Ferrous low spin derivatives. *Biochemistry.* 15:378–387.

Potter, W. T., J. H. Hazzard, M. G. Choc, M. P. Tucker, and W. S. Caughey. 1990. Infrared spectra of carbonyl hemoglobins: characterization of dynamic heme pocket conformers. *Biochemistry.* 29:6283–6295.

Shiga, T., K. Hwang, and I. Tyuma. 1969. Electron paramagnetic resonance studies of nitric oxide hemoglobin derivatives. I. Human hemoglobin subunits. *Biochemistry.* 8:378–383.

Wajnberg, E., G. Bemski, L. J. El-Jaick, and O. C. Alves. 1996. Nitrosyl hemoglobins: EPR above 80 K. *Int. J. Biol. Macromol.* 18:231–235.

Wajnberg, E., M. P. Linhares, L. J. El-Jaick, and G. Bemski. 1992. Nitrosyl hemoglobin: EPR components at low temperatures. *Eur. Biophys. J.* 21:57–61.