CBPF-NF-019/95

Nitrosyl Hemoglobins: EPR Above 80K

E. Wajnberg, G. Bemski, L. Jaccoud El-Jaick and O. Cambraia Alves*

Centro Brasileiro de Pesquisas Físicas - CBPF
Rua Dr. Xavier Sigaud, 150
22290-180 - Rio de Janeiro-RJ, Brasil

Permanent address: Instituto de Química, Universidade Federal Fluminense, Outeiro
São João Batista, 24020-150, Niterói/RJ,Brasil

abstract

The EPR spectra of nitrosyl hemoglobin and myoglobin in different conditions (native, denatured and lyophilized), as well as of hematin-NO were obtained in the temperature range of 80K-280K. There is a substantial and reversible decrease of the areas of the EPR spectra of all the hemoglobin samples above 150K. The interpretation of the results implies the existence of two conformational states in thermal equilibrium only one of which is EPR detectable. Thermodynamical parameters are determined for the hexa and penta-coordinated cases.

Key-words: Nitrosyl hemoproteins; EPR.

1 Introduction

The important role of the sixth ligand in hemoglobin (Hb), particularly of the physiologically important oxygen, has lead to a large number of studies which use physical techniques.

It has been known for a long time that Fe exists in Fe^{2+} or Fe^{3+} state. The physiologically important Fe^{2+} is in general not accessible to studies by Electron Paramagnetic Resonance (EPR), hence the EPR effort has concentrated on hemoglobins in Fe^{3+} state with ligands such as H_2O, F, CN, etc. Nitric oxide (NO) is an exception. When liganded to the sixth position of the heme iron the latter is ferrous with S=O, while the total spin of the unit (S=1/2) originates from NO. The protein becomes paramagnetic easily observed and widely studied by EPR (1).

Experiments with HbNO have shown spectral differences for NO bound to the α and β chains of the tretameric hemoglobin, as well as differences between tense (T) and relaxed (R) conformations (2-9). There are nevertheless still some questions concerning the electronic structure and conformation of the protein with NO ligand (10-11).

We have previously noticed spectral changes of the EPR absorption as a function of temperature below 100K (11). In this paper we describe an extension of these studies to higher temperatures using different samples of HbNO, MbNO and hematin-NO.

2 Experimental Procedure

Hb was prepared from fresh human blood using standard procedures. Mb (horse heart, Sigma) was completely oxydized with $K_3Fe(CN)_6$ and the excess was removed by gel filtration. Powder Hb was obtained with a Labconco lyophilizer 75200. Hematin was reduced with excess (2:1) sodium dithionite and kept in anaerobic conditions with N_2 flux. Solutions were 3 to 5 mM of heme in phosphate buffer, 0.1 M, pH 6.5, except for hematin which was dissolved in pyridine. All samples were transferred to the EPR tubes which contained $MgCr^{2+}$ as g-marker, in the presence of a nitrogen (N_2) atmosphere. The EPR sealed tubes were alternately evacuated and purged with N_2 by means of a syringe, except for the powder samples. In the latter case this procedure was performed with the powder samples in the tube.

HbNO and MbNO complexes in saturated conditions (HbNO in R conformation) were prepared deoxygenating the samples with N_2 flux followed by injection of NO gas. HbNO in the T conformation was obtained in the presence of inositolhexaphosphate (IHP), two per heme. 10% in volume of a R HbNO sample was added to the previously deoxygenated Hb-IHP sample. Samples were used at least one hour after preparation, time sufficient for distribution of NO among the four polypeptide chains.

The 75% glycerol sample was prepared as for the R conformation after dilution of the Hb solution with glycerol 3:1 (glycerol:solution). Powder HbNO was obtained injecting NO gas into the previously purged tube. Heat denatured HbNO and MbNO were prepared as in (12), (80^0C, 5 hours).

EPR experiments were performed with an E-9 Varian X-band spectrometer. Measurements between 80K and 280K were performed with a N_2 flux system. The temperature was measured with a chromel vs constant thermocouple located just above the sample.

3 Results

Fig. 1 presents the EPR spectra of the five types of HbNO samples examined. All of them have been previously observed (5-8,12). The vertical lines in fig. 1 indicate the positions at which amplitudes of the lines were measured for analysis. Intensities at other points, as well as the double integral of the spectra (areas) were also measured, yielding identical temperature dependence. Figure 1a shows the spectrum in R conformation and is identical to the "high" temperature, C component previously described (11). The 75% glycerol sample (fig. 1b) gives a very similar spectrum, but for the absence of a slight indication of the three line hyperfine interaction present in fig. 1a. Nitrogen hyperfine interaction, characteristic of the five-coordinated iron (5) is clearly seen in the T conformation in the powdered and denatured samples (fig. 1c, 1d and 1e, respectively). The presence and absence of the hyperfine structure have been attributed to changes in the distance between Fe and the nitrogen ligand of the proximal histidine (5).

The analysis of the HbNO spectra is simplified by the temperature range of the present experiments. While at low temperatures (<100K) saturation effects had to be considered (11) at higher temperatures these effects are not important since the shapes of the spectra remain unchanged, independent of the klystron power. Their widths remain constant, while their intensities increase with power level without indication of saturation. This observation was confirmed for all HbNO and MbNO samples. We adopted in consequence a 20 dB level ($\sim$ 2mW) in all experiments.

In fig. 2 and 3 we compare the temperature dependence of the intensities of the spectra, proportional to the number of spins. The spectra of the g-marker are used to verify that the effect of changes of the Q factor of the cavity with temperature is negligible. Except for hematin-NO and native MbNO (fig. 3) the curves show a sharp decrease of intensities which starts between 150K and 220K. These results were independent of the heating (or cooling) cycles which were varied in diverse ways. In a typical experiment a sample prepared at room temperature was cooled either to the lowest temperature (80K), or to a temperature slightly below 273K. The temperature was then increased (decreased)

by about 5 degree intervals and the spectra taken after 5 minutes equilibration. The temperature cycles were perfectly reproducible. In all cases the maximum temperature was above 273K.

4 Discussion

Spectral changes are negligible for temperatures above 80K, except for hematin-NO whose area however remains constant.

The most interesting feature of the results is the pronounced and reversible decrease of the intensities (or areas) of the spectra of the different nitrosyl hemoglobins above about 150K (fig. 2). Deviation from linearity at higher temperatures (about 230K) have been attributed to the effect of the thawing of hydration waters (13). This is not the effect observed here since it occurs, in powdered, lyophilized as well as in glycerol samples, thus eliminating problems which arise from the aggregation of solvent on freezing.

Since there is no change in the shape of the EPR spectra of the samples we can assume the existence of higher lying electronic energy levels.

In the case of metHb (Fe^{3+}, S=5/2) a decrease of amplitude of the signal with increasing temperature is due to zero field splitting of the lowest energy level ($\pm 1/2$, $\pm 3/2$, $\pm 5/2$) (17). Nevertheless this possibility is normally rare in the Fe^{2+} heme proteins because of the large energy separation between the lowest and the first excited state. Indeed such a model failed to fit the experimental curves.

Differences in the temperature behavior are also observed in the Lamb-Mössbauer factor (14-16), but they point invariably to a decrease of the excess $< x^2 >$ above 200K in lyophilized samples as compared to crystals or frozen solutions. Such differences do not appear in our samples (fig. 2). Hence the loss of flexibility of the segment of protein which involves the heme and NO, important as it is in Mössbauer results does not express itself here.

The experimental curves can be fitted with an expression of the form:

$$I = I_0/T(1 + exp(-\Delta G/kT)),$$

where ΔG is the free energy difference between two conformational states, I_0 is a normalization factor and 1/T is the factor associated to the high temperature limit of the Boltzmann distribution of the Zeeman doublet dominant at low temperatures.

Such a fit suggests a thermal equilibrium with a second species not detectable by EPR, corresponding to slight changes in the geometry of the heme, the proximal histidine and the ligand NO. Considering $\Delta G = \Delta H - T\Delta S$ with ΔH and ΔS temperature independent, we obtain $\Delta H = 29.3 \pm 0.9$ kJ/mol and $\Delta S = 105 \pm 6$ J/molK for R type

samples, and $\Delta H = 12 \pm 2$ kJ/mol and $\Delta S = 42.4 \pm 0.8$ J/mol for five coordinated samples. Results are shown in fig. 2.

Perutz has assigned two species in thermal equilibrium in both R and T states of azide carp Hb to two stereochemical configurations differing in their Fe-N_{porph} distances, combined with the rigidity of Fe atom below 220K (20,21). Our experimental results evidence effects even at lower temperatures with differences in the behavior of those two quaternary structures. Our ΔH values suggest that stereochemical changes are favored in the five coordinated conformations which have longer Fe-N (proximal histidine) distances. The values for ΔH are of the same magnitude as the ones determined for horse Mb (18) and for erythrocruorin between 100K and 310K (19), respectively. Our results differ from the previously reported ones in that they show no evidence of spectral shape changes.

Waleh et all have shown (10) that in the nitrosyl ferrous heme compounds the electronic energy diagram depends on the local geometry around the heme and can vary from strong spin localization on NO (the situation "normally" observed by EPR in heme proteins) to one with the spin localized on the iron d_z^2 orbital. Minor variations in ligand distances, or in bonding angles affect the energy differences and the relative orders of the levels. Such spin shifts could be caused by steric factors resulting from temperature increase and a shift to Fe^{2+} can reflect itself in an increase of the relaxation rates leading to a line broadening beyond the possibility of the EPR detection.

MbNO does not show the sharp decrease of the EPR signal intensity. This anomalous behavior of MbNO indicates a stabilization of a species as in cytochrome c oxidase (18) and in Aplysia brasiliana (19). In the latter the distal histidine is absent having a strong effect on the eletronic structure of the complex. Such a difference in histidine binding between Hb and Mb was also observed in met-H_2O complexes despite their very similar environments (22). Other unexpected experimental results have also been observed in MbNO, such as the complexity of the kinetics shown by the non-exponential rebinding after photodissociation at room temperature (22).

Photolysis of HbNO at low temperatures also results in the loss of EPR signal (23) accompanied by a slight displacement of NO, similar to transitions between A and B states observed in MbCO (24). The present experiments indicate existence of energy barriers which are somewhat higher, but of the same order of magnitude as in those experiments.

The existence of various conformational species in equilibrium is the principal aspect of the substates model (25). Our observations indicate that this is a general property of the hemoglobins, independent of their quaternary structure, or solvent conditions.

Acknowledgements

The authors are thankful to Elena Mavropoulos for technical assistance, Instituto de Hematologia (Rio de Janeiro, Brazil) for blood samples and CNPq for partial support of this work.

5 Figure Captions

Figure 1 - EPR spectra of different human HbNO samples at 80K; (a) R conformation, (b) 75% glycerol, (c) T conformation, (d) powdered sample, (e) denatured.

Figure 2 - The temperature dependence of the intensities of EPR spectra of Hb: (a) powdered, (b) R conformation, (c) T conformation. The solid lines are examples of the theoretical fits using the values of free energies and entropies given in the text.

Figure 3 - The temperature dependence of the intensities of EPR spectra: (a) g-marker ($MgCr^{2+}$), (b) hematin-NO, (c) native MbNO, (d) denatured MbNO, (e) native HbNO, (f) denatured HbNO.

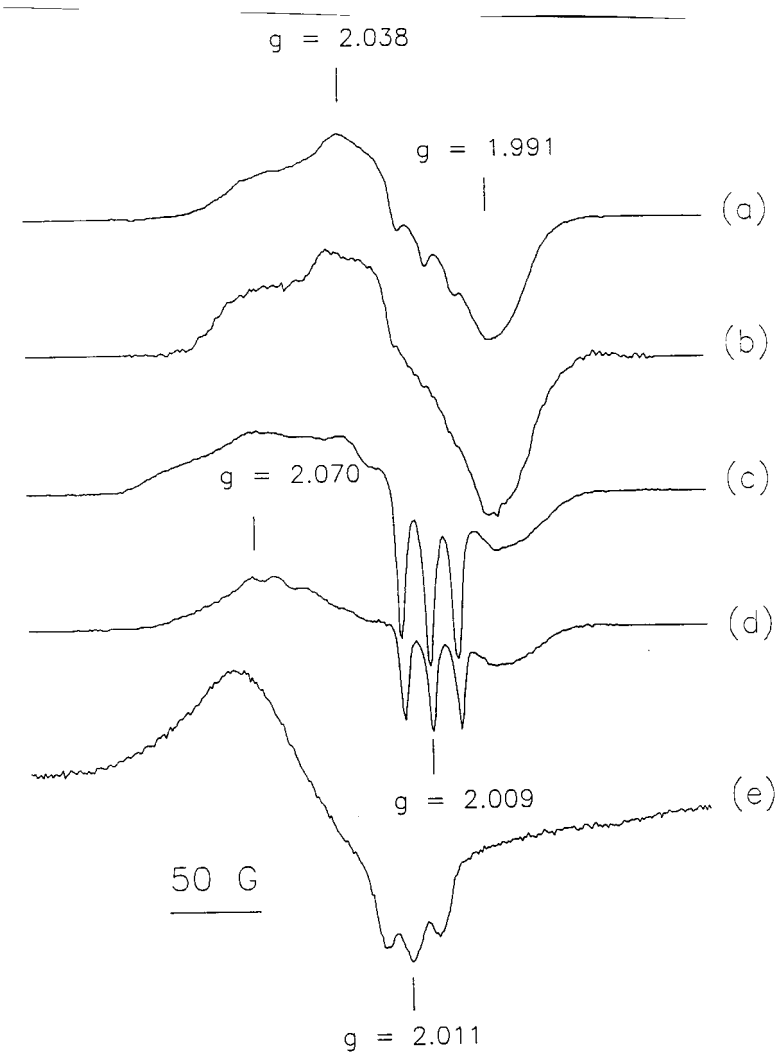

Fig. 1

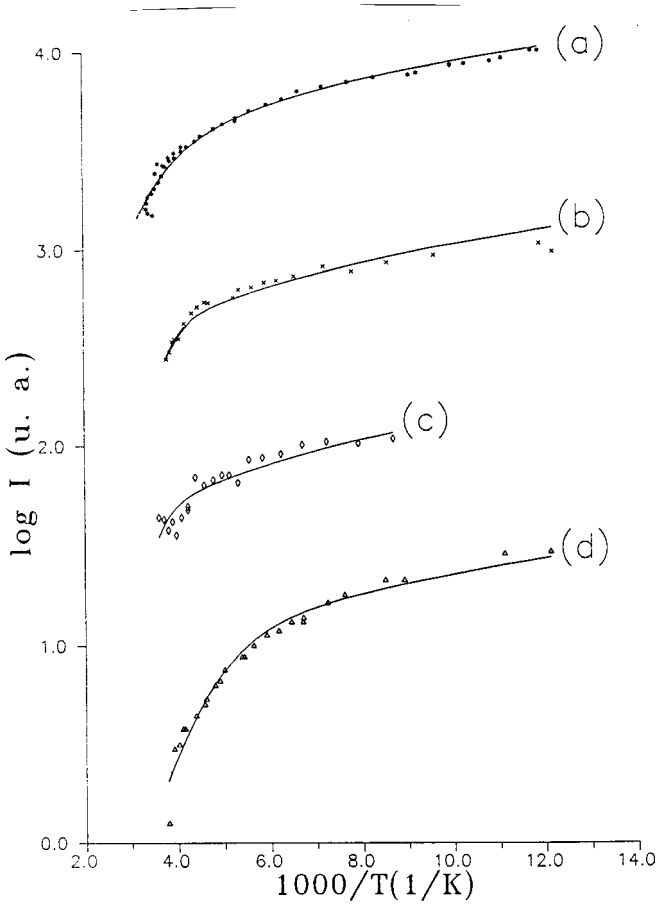

Fig. 2

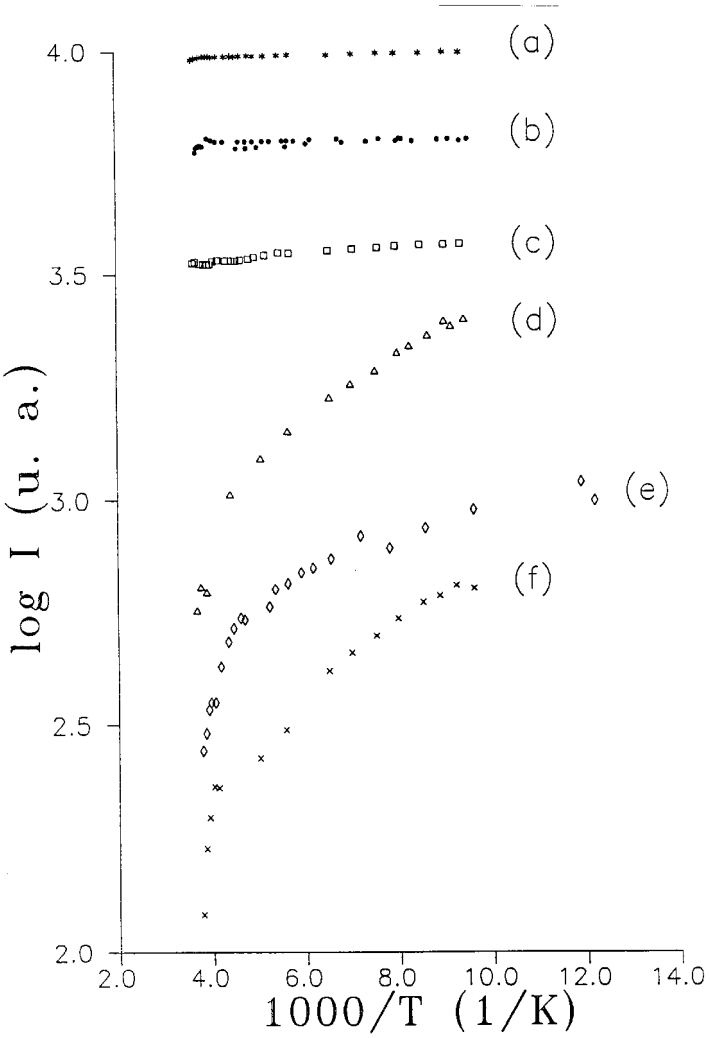

Fig. 3

References

[1] Ducrocq, C.; Henry, Y.; Drapier, J.C.; Servent, D.; Pellat, C.; Guissani, A.; Eur. Biophys. J. , 1991, 20, 1.

[2] Shiga, T.; Hwang, K.; Tyuma, I.; Biochem. 1969, 8, 378.

[3] Chien, J.C.W.; J. Chem. Phys. 1969, 51, 4220.

[4] Rein, H.; Ristau, O.; Scheler, W.; FEBS Lett. 1972, 24, 24.

[5] Szabo, A.; Perutz, M.F.; Biochem. 1976, 15, 4427.

[6] Taketa, F.; Antholine, E.E.; Chien, J.Y.; J. Biol. Chem. 1978, 253, 5448.

[7] Mun, S.K.; Chang, J.C.; Das, T.P.; Proc. Natl. Ac. Sci. USA, 1979, 76, 4842.

[8] Louro, S.R.W.; Ribeiro, P.C.; Bemski, G.; Biochim. Bioph. Acta. 1981, 670, 56.

[9] Hutterman, J.; Burgard, C.; Kappi, R.; J. Chem. Soc. Faraday Trans. 1994, 90, 3077.

[10] Waleh, A.; Ho, N.; Chantranupong, L.; Lowe, G.H.; J. Am. Chem. Soc. 1989, 111, 2767.

[11] Wajnberg, E.; Linhares, M.P.; El-Jaick, L.J.; Bemski, G.; Eur. Biophys. J. 1992, 21, 57.

[12] Alves, O.C.; Wajnberg, E.; Int. J. Biol. Macromol. 1993, 15, 273.

[13] Wittenberg, B.A.; Briehl, R.W.; Wittenberg, J.B.; Biochem. J. 1965, 96, 363.

[14] Kurinov, I.V.; Krupyanskii, Yu.F.; Genkin, M.V.; Davydov, R.M.; Suzdalev, V.I.; Goldanskii, V.I.; Biophysics 1988, 33, 436.

[15] Engelhard, M.; Kohl, K.D.; Muller, K.H.; Hess, B.; Heidemeier, J.; Fischer, M.; Parak, F.; Eur. Biophys. J. 1990, 19, 11.

[16] Frolov, E.N.; Fischer, M.; Graffweg, E.; Mirishly, M.A.; Goldanskii, V.I.; Parak, F.; Biophys. J. 1991, 19, 253.

[17] Feher, G.; Electron Paramagnetic Resonance with Applications to Selected Problems in Biology. Gordon and Breach publish. 1969, 62.

[18] Morse, R.H.; Chan, S.I.; L. Biol. Chem. 1980, 255, 7876.

[19] Caracelli, I.; Meireless, N.; Tabak, M.; BafiaFilho, O.; Nascimento, O.; Biochem. Biophys. Acta 1988, 955, 315.

[20] Messana, C.; Cerdonio, M.; Shenkin, P.; Noble, R.; Fermi, G.; Perutz, R.N.; Biochem. 1978, 17, 3652.

[21] Perutz, M.F.; Nature, 1992, 358, 548.

[22] Steinhoff, H.J.; Lieutenant, K.; Redhardt, A.; Biochim. Bioph. Acta 1989, 996, 49.

[23] Linhares, M.P.; El-Jaick, L.J.; Bemski, G.; Wajnberg, E.; Int. J. Biol. Macromol. 1990, 12, 59.

[24] Mourant, J.R.; Braunstein, D.P.; Chu, K.; Frauenfelder, H.; Niehaus, V.; Ormas, P.; Young, R.D.; Biophys. J. 1993, 65, 1497.

[25] Austin, R.H.; Beeson, K.W.; Eisenstein, L.; Frauenfelder, H.; Gunsalus, I.C.; Biochem. 1975, 14, 5355.