JOURNAL OF MAGNETIC RESONANCE, Series B **113,** 119–124 (1996)
ARTICLE NO. 0164

Spin–Lattice Relaxation of Denatured Nitrosyl Hemoproteins

ELIANE WAJNBERG* AND ODIVALDO CAMBRAIA ALVES†

Centro Brasileiro de Pesquisas Físicas, Rua Xavier Sigaud, 150, 22290-180 Rio de Janeiro, Brazil

Received May 14, 1996; revised August 12, 1996

The temperature dependence of the spin–lattice relaxation of denatured nitrosyl hemoglobin (HbNO), nitrosyl myoglobin, powdered HbNO, and hematin-NO was studied between 4 and 70 K. The results were fitted with both T^n and $e^{-\Delta/T}$ models. In the first model, the relaxation is mediated by tunneling modes of a two-level system. A correlation between the n values and the functional state of the protein was observed. The striking coincidence of the range of the low-lying energy level and the temperature range where EPR spectra change suggests the existence of two conformations of the bound heme. The importance of the presence and structure of the globin is revealed in the difference between relaxation parameters of native proteins, denatured proteins, and hematin. © 1996 Academic Press, Inc.

INTRODUCTION

The observed temperature dependence of the spin–lattice relaxation (SLR) time, T_1, of metalloproteins is of the T^n, Raman type with n, however, related to the fractal dimension of the polymers (*1*). Relaxation of denatured azide methemoglobin has given further support for the effect of conformational changes on the relaxation process (*2*). More recently, iron in nitrosyl hemoproteins has been the subject of interest in relaxation studies (*3–6*).

It was first observed that the T_1 value of nitrosyl myoglobin (MbNO) in solution at low temperatures (4.2 to 20 K) shows a linear temperature dependence. No attempt was made to identify the relaxation mechanism (*4*). More recently, measurements in solution and on powdered samples over a wide temperature range have shown an anomalous T^n power law with $n = 2.2 \pm 0.3$ (*5*). This result was attributed to tunneling within localized states (*7, 8*) associated with the existence of conformational equilibrium in hemoproteins.

The power-saturation studies between 1.4 and 4.2 K and the temperature-dependence above 80 K of EPR spectra of nitrosyl hemoglobin (HbNO) crystals have shown a T_1 and spin–spin relaxation time, T_2, behavior that was explained qualitatively by a relaxation process which involves heme–heme magnetic dipolar interactions between both kinds of subunits (*3*). On the other hand, electron paramagnetic resonance (EPR) spectra of HbNO solutions studied in the temperature range from 7.5 to 104 K have been shown to be composed of at least three components (A, B, and C) whose intensities have different dependences on temperature and microwave power level (*6*). Their relaxation behavior was studied by the continuous saturation method, and the temperature dependence of T_1 of A, the low-temperature and low-power-level component, was shown to follow an Orbach-like mechanism with a characteristic energy of $\Delta = 28$ cm^{-1}. It was proposed that this energy refers to a difference between two different geometries of bound heme.

Nitrosylhemoproteins do not exhibit a Raman relaxation mechanism with a fractal exponent, but they are interesting, providing information on two-level systems. These results stimulated the investigation of the SLR mechanism of denatured hemeproteins, in search of information on excitations and dynamics of their disrupted structure which has no physiological function.

This work aims to verify the effect of protein denaturation, as well as the importance of the presence of globin on the SLR mechanism. The present paper is a continuation of the studies on the temperature dependence of the SLR of hemoproteins and involves observation of heat-denatured MbNO and HbNO, powdered HbNO, and nitrosyl hematin (hematin-NO).

EXPERIMENTAL

Hb was prepared from fresh human blood using standard procedures. Mb (horse heart, Sigma) was completely oxidized with $K_3Fe(CN)_6$ and the excess was removed by gel filtration. Hematin (Sigma) was reduced with excess (2:1) sodium dithionite and kept under anaerobic conditions with a N_2 flux. Solutions were 1 to 2.5 m*M* heme in phosphate buffer 0.1 *M,* pH 7 except for hematin which was dissolved in pyridine. Nitrosylation was obtained by introducing nitric oxide (NO) gas directly into the previously deaerated EPR sample tube. Heat-denatured MbNO and HbNO were prepared at 80°C for 5 h (*9*). Powdered samples were prepared using a Labconco 75200 lyophilizer.

* To whom correspondence should be addressed.
† Instituto de Quimica, Universidade Federal Flumininse, Outeiro de São João Batista s/n, Niteroi, Brazil.

1064-1866/96 $18.00
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

EPR measurements were performed in a E-9 Varian X-band spectrometer from 4 to 100 K, using a helium-flow cryostat (Helitran LTR-3-110). Temperatures were measured with a Au–Fe vs chromel thermocouple placed below the sample.

The half-saturation power, $P_{1/2}$, and the SLR rate T_1^{-1} were estimated as previously described, using the continuous-saturation method (*10, 11*). Although this is not the best method for obtaining absolute values of T_1, the continuous-wave-saturation method is adequate to obtain the temperature dependence and relative values of T_1. It is commonly used in biological systems (*12, 13*). This method is not strictly applicable for determination of the relaxation times of systems with dipolar coupling but it can still be used to estimate the magnitude of the dipolar-relaxation enhancement (*14*). A value of the inhomogeneity parameter, b, of less than 1 suggests a dipolar interaction. Only hematin samples have b values slightly below 1 and a low degree of saturation as defined by Galli *et al.* (*14*). For these samples, the fits were performed assuming that they were the inhomogeneous limit ($b = 1$). In such a case, the dipolar interaction adds to the spin-relaxation mechanism as in (*14*). The intensities of the $g = 2.07$ and $g = 2.011$ EPR lines in denatured samples and the $g = 2.07$ signal in hematin were monitored as indicated in the spectra of Fig. 1.

The saturation conditions do not allow for the determination of $P_{1/2}$ at temperatures higher than 70 K. Thus, in the temperature range from 4 to 70 K, the dependence of T_1^{-1} was fitted to either one of the expressions

$$T_1^{-1}(\text{arbitrary units}) = AT^n + C \qquad [1]$$

$$T_1^{-1}(\text{arbitrary units}) = B \exp\left(-\frac{\Delta}{T}\right) + D, \qquad [2]$$

where n, A, B, Δ, and D are fitting parameters.

RESULTS AND DISCUSSION

The EPR spectra were obtained as a function of temperature and microwave power. In Fig. 1, the EPR spectra of denatured MbNO and HbNO, and powdered HbNO at 6 K and 20 dB ($\simeq$1.3 mW) are presented. The spectra are asymmetric and show a well-resolved three hyperfine line, a structure characteristic of the penta-coordinated iron (*15*). We observed that the spectral shape does not depend either on temperature or on microwave power.

EPR spectra of hematin-NO as a function of temperature at 22 dB are shown in Fig. 2. Above 77 K, the spectrum undergoes a change in shape similar to that observed in native HbNO at lower temperatures (about 30 K) (*6*). Below 77 K, the spectra do not depend on the temperature and have a negligible dependence on microwave power (spectra not shown). At these temperatures, only the high-field line at $g = 2.07$, which has a smaller contribution from other components, was used for saturation measurements.

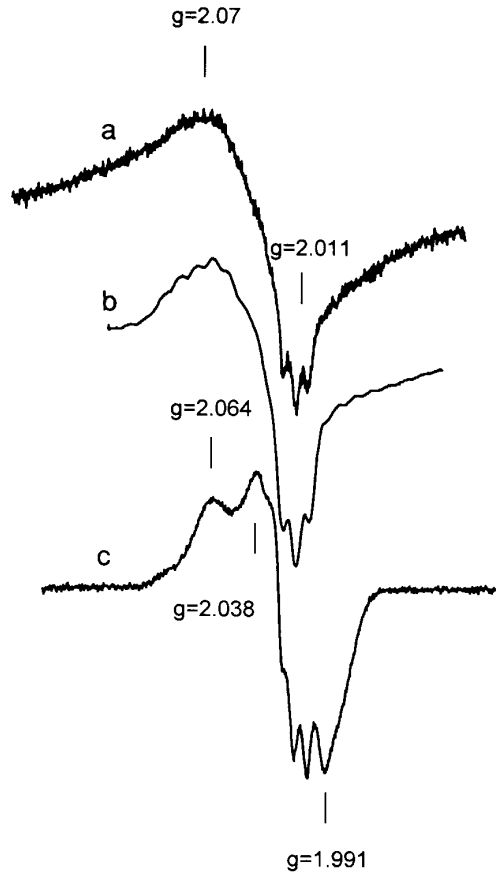

FIG. 1. EPR spectra of different samples at 6 K and 20 dB; (a) denatured MbNO, (b) denatured HbNO, and (c) powdered HbNO.

In Fig. 3, the temperature dependence of the SLR rates for denatured HbNO (Fig. 3a), denatured MbNO (Fig. 3b), powdered HbNO (Fig. 3c), and hematin-NO (Fig. 3d) is presented. The solid and dashed lines correspond to the best fits of Eqs. [1] and [2], respectively. It is seen that, except for hematin-NO, both fits to the experimental data are equivalent within the experimental error. Analysis of the residual differences between experimental and calculated values confirms that the two fits are actually undistinguishable (Fig. 4). Only hematin-NO is better fitted by the T^n function (Fig. 4d).

For comparison, the parameters of these fits are listed in Table 1, together with those from other references (*5, 6*).

Table 1 shows a T^n dependence for the SLR rate with $n = 2.2 \pm 0.2$ for native and powdered samples except for the HbNO solution. In the denatured samples and hematin, this exponent increases to 3.8 ± 0.4. This temperature dependence is associated with a model based on relaxation of a two-level system mediated by tunneling modes. The TLS (tunneling localized state) proposed for powdered MbNO

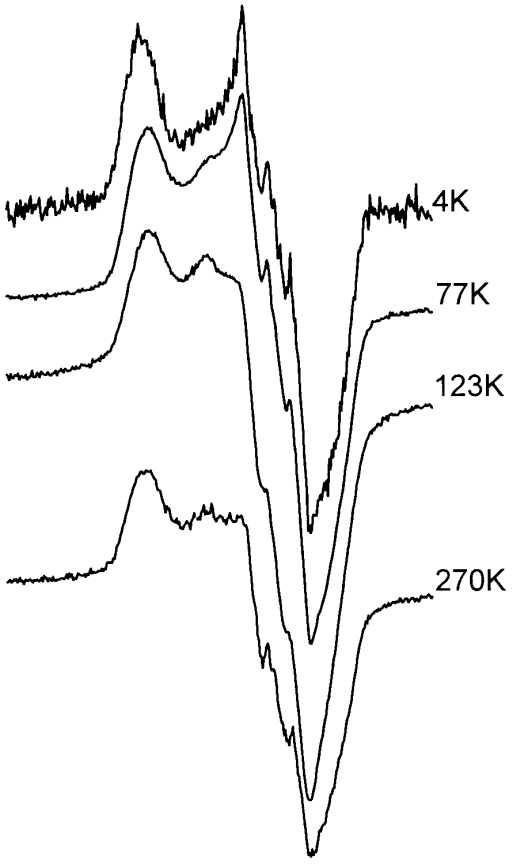

FIG. 2. EPR spectra of hematin-NO as a function of temperature at 22 dB.

(*5*) can be extended to powdered HbNO, since frozen solutions of this complex also exhibit more than one binding conformation associated with the two-level system (*6, 16*).

In this model, the value of n is given by $n = 2 + \lambda$, where λ is a parameter that relates the density of states, $\rho(E)$, and the energy, E, by the expression $\rho(E) \approx E^{\lambda}$; λ is related to the value of the characteristic energy E_{max}, the maximum energy of TLS, or the cutoff in the distribution of the asymmetry parameter of the double-well potential. There is a correlation between the λ values obtained and the functional state of the protein. Native and powdered samples, which are reversible to native states, present low λ values (0.2 to 0.4) when compared to the values of denatured proteins and hematin or to the values of 1.5 to 1.6 of inorganic amorphous materials (*7*).

From Eq. [2], one obtains evidence for a low-lying energy level of 65 cm^{-1} for denatured HbNO, 51 and 77 cm^{-1} for denatured MbNO, and 75 cm^{-1} for hematin-NO. These values are higher than those obtained for the low-power component of native HbNO, 28 cm^{-1} (*6*), but they are still much lower than the difference in energy between the ground and excited electronic states in the proposed energy level diagram of nitrosyl hemoproteins (*17*).

It is interesting to observe that native HbNO shows a low-lying energy level of 28 cm^{-1} for the A component and its EPR spectra change at about 32 K (*6*), when the B component (low-temperature and high-microwave-power component) disappears and another component called C appears. Similarly, our results indicate a low energy level of 74 cm^{-1} for hematin-NO with EPR spectral changes at temperatures about 80 K.

Waleh *et al.* have shown that the observed variations in EPR spectra for hemeproteins from various sources and under different experimental conditions can be accounted for in terms of changes in the ligand geometry (*18*). In that case, the values determined from SLR and the temperature dependence of the EPR spectra can be related to a difference between two conformations (geometries) of the bound heme, produced by different axial ligand distances, or Fe–N–O bond angles. The energy values are in good agreement with the low-frequency modes (10–100 cm^{-1}) observed in the external medium of Mb by inelastic neutron scattering (*19, 20*). Thus, the variation in the ligand geometry could be induced by these phonons.

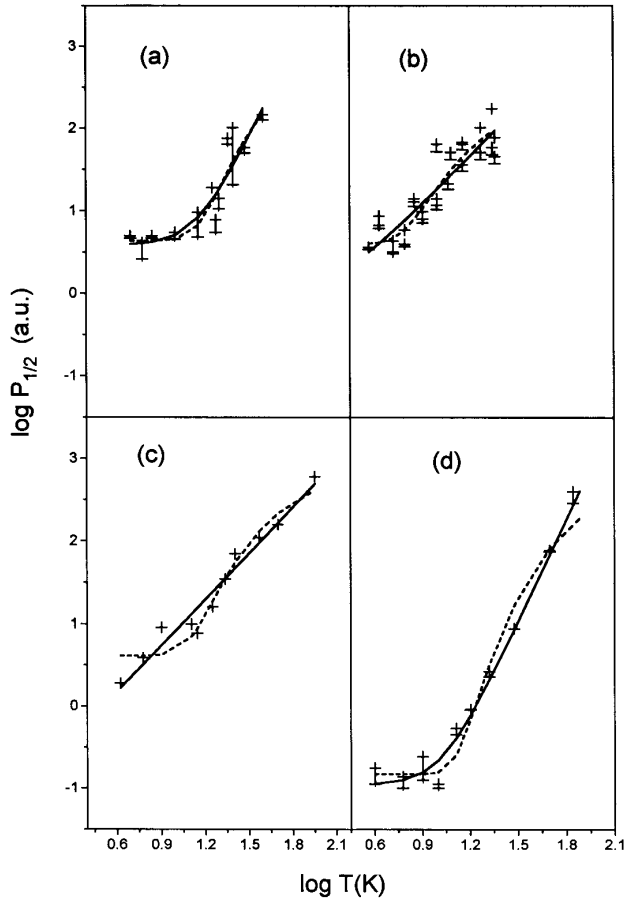

FIG. 3. The temperature dependence of the SLR rates of $g = 2.07$ for (a) denatured HbNO, (b) denatured MbNO, (c) powdered HbNO, and (d) hematin-NO. ---, T^n, and —, $e^{-\Delta/T}$ models.

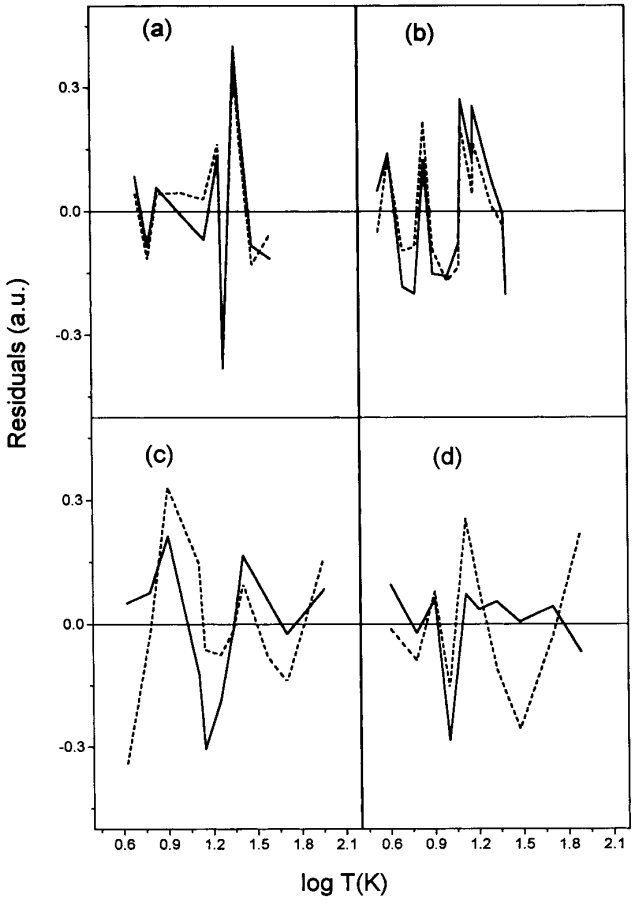

FIG. 4. Residual differences between experimental and ---, T^n, and —, $e^{-\Delta/T}$ models for SLR rates: (a) denatured HbNO, (b) denatured MbNO, (c) powdered HbNO, and (d) hematin-NO.

Although denatured HbNO and MbNO and powdered HbNO do not show variations in their EPR spectra, the observed low-lying energy state suggests the existence of a second conformational state not detectable by EPR. This hypothesis is in agreement with the anomalous temperature dependence of the intensity of EPR spectra of these complexes which was attributed to the equilibrium between two different conformations (*16*), one of which is EPR silent.

The λ values obtained above for the TLS model correlate with the energy difference between the conformations at equilibrium. A higher cutoff of the asymmetry parameter is associated with a higher low-lying energy state.

In view of the fact that Eq. [1] fits almost all samples analyzed in Table 1, we estimate the values of T_1 using this equation. Equation [2] would not yield significantly different results. The values of T_1 (s) can be estimated considering the linewidth and the Q factor of the cavity (*11, 21*) and are in good agreement with those obtained by pulsed methods (*5*). Table 2 presents the data for T_1^{-1} (s^{-1}) at three different temperatures. The contributions of the two terms of Eq. [1] were calculated separately.

These results, supported by Fig. 3, show that at temperatures lower than 11 ± 3 K the principal contribution to the relaxation of denatured and hematin samples is that from the constant term. This term was not observed either in native or in powdered NO hemoproteins (*5, 6*). This behavior could be explained by considering that, in the unfolded structure of denatured samples and in hematin, the interheme distances are sufficiently short to allow the spin–lattice relaxation to be dominated at low temperatures by cross relaxation between hemes.

Cross relaxation is possible only if the transitions have overlapping lines (*22*). The cross-relaxation rate T_{1c}^{-1} modulated by dipole interaction can be calculated using the values obtained by Bloembergen *et al.* for the overlap of Gaussian resonance lines (*23, 24*)

$$T_{1c}^{-1} = \frac{g^3\mu_B^3}{2\pi^{1/2}\hbar Hr^6}\,[S(S+1)(1-3\cos^2\theta)]^2, \quad [3]$$

TABLE 1
Fitting Parameters of T_1^{-1} According to Eqs. [1] and [2]

	g	A	n	C	B	Δ (cm^{-1})	D	Ref.[a]
Native HbNO	—	—	—	—	$10^{5.1}$	28	—	*6*
Powdered HbNO	2.064	0.11	1.9	—	928	51	4.2	This work
	2.003	0.048	2.2	—	1120	47	2.3	This work
Denatured HbNO	2.07	0.0003	3.6	3.9	1566	65	4.4	This work
	2.011	0.0002	3.6	2.0	785	65	2.3	This work
Native MbNO	2.007	—	2.4	—	—	—	—	*5*
Powdered MbNO	2.064	—	2.4	—	—	—	—	*5*
	2.003	—	2.1	—	—	—	—	*5*
Denatured MbNO	2.07	0.003	3.5	3.8	618	51	4.4	This work
	2.011	0.00001	4.5	1.3	1836	77	1.0	This work
Hematin-NO	2.07	0.00015	3.2	0.2	115	76	0.2	This work

[a] In Refs. (*5, 6*), only the best fitting was considered and the constant term was not observed.

TABLE 2
Absolute Values of T_1^{-1} (s^{-1}) According to Eq. [1]

	$AT^n \times 10^6$	$C \times 10^6$	$T_1^{-1} \times 10^6$
	Hematin-NO—$g = 2.07$		
5 K	0.4	1.4	1.8
10 K	2.1	1.4	3.5
15 K	7.8	1.4	9.2
	Denatured MbNO—$g = 2.07$		
5 K	5.9	25	30.9
10 K	69	25	94
15 K	288	25	313
	Denatured HbNO—$g = 2.07$		
5 K	0.6	24	24.6
10 K	7.1	24	31.1
15 K	30	24	54

where μ_B is the Bohr magneton, $\hbar$ is Planck's constant, r is the mean distance between heme groups, H is the magnetic field, and θ is the angle between the radius vector between two neighboring sites and the magnetic field. Using the value of the constant term from Table 2 in Eq. [3], we estimate the distance between heme groups as 21 Å for hematin-NO and 12 Å for denatured HbNO and MbNO.

In the R structure, the distance between iron atoms of the α chains is 36 Å and between iron of the β chains is 33 Å, while in the T structure the corresponding distances are 35 Å and 40 Å (*25*). A decrease of the distance between heme groups in denatured and hematin-NO samples as compared to the values in the R or T structure of HbNO, together with the total overlap between the spectral functions, makes cross relaxation possible.

From volume changes, we can roughly estimate the distances between hemes due to denaturation and dehydration. Dehydration decreases the volume by a factor of at least 50 while this factor is 2 for denaturation. The shortest mean distance between molecules because of dehydration is about 30 Å, which is similar to the interheme distance in hemoglobin. As cross relaxation is only observed in denatured samples, it indicates that a drastic change in the protein structure occurs during the denaturation process which leaves the heme groups in closer contact in denatured than in powdered samples, shortening, for example, the heme distance to the globin surface. It was observed in metHb that denaturation induces one more structural change, distorting hemichrome P induced by dehydration into P′, which involves displacements and torsions of the F helix, of the surface of the protein (*9*). For hematin, the mean distance between heme groups could be shorter than that estimated from the concentration of the solution, 86 Å, since dimer formation has been suggested (*26*).

It has been pointed out that the effect of dehydration of MbNO is to increase the SLR rate from 5.6×10^3 s^{-1} in solution to 1.8×10^4 s^{-1} in powdered MbNO at 20 K (*5*). This effect is increased in HbNO as seen by the increase of the SLR rate from 1.6×10^4 s^{-1} in solution to 1.8×10^8 s^{-1} (at least in powdered HbNO). We observed that the SLR rate increases further in denatured MbNO relative to powdered MbNO, an effect which is also observed in the equivalent HbNO samples. The values of T_1^{-1} at 20 K are 1.1×10^8 and 7.9×10^8 s^{-1} for denatured MbNO and HbNO, respectively. This indicates that the denaturation process can go further than dehydration, as previously observed in metHb (*9*).

Both models seem to be able to explain our results. The exponential model gives information about the influence of neighbors on the SLR mechanism and suggests a relation between the low-lying energy level and changes of conformation in bound heme. On the other hand, in the tunneling localized states model, the importance of structure–function relationship is observed and the similarity between proteins and glasses is strengthened. In the T^n temperature dependence of the SLR Raman rate in low-spin Fe(III) proteins, the n value was at first interpreted in terms of the fractal dimensions of the proteins (*1*). More recently, studies of copper as well as iron proteins have shown that the relaxation behavior is not correlated with the fractal dimensions of a protein (*27*). The similarity in the n value of the Raman rate for low-spin Fe(III) in heme proteins and in small molecules, such as the low-spin Fe(III) porphyrin, suggests that n is determined rather by the local heme environment than by the long-range structure of the protein (*27*). Nevertheless, our results show that in nitrosyl hemoprotein, relaxation processes depend on the presence and structure of globin, as shown by its effect on the characteristic exponents of the temperature dependence. The NO ligand has proved to be more sensitive than low-spin complexes to the effects of the conformational equilibrium of hemoproteins.

ACKNOWLEDGMENT

The authors thank Dr. G. Bemski for his critical reading of the paper.

REFERENCES

1. J. T. Colvin and H. J. Stapleton, *J. Chem. Phys.* **82,** 4699 (1985).
2. E. Wajnberg and G. Bemski, *Phys. Lett. A* **132,** 4 (1988).
3. D. C. Doetschman and S. G. Utterback, *J. Am. Chem. Soc.* **103,** 2847 (1981).
4. P. J. Muench and H. J. Stapleton, *J. Chem. Phys.* **82,** 2828 (1985).
5. O. R. Nascimento, L. M. Neto, and E. Wajnberg, *J. Chem. Phys.* **95,** 2265 (1991).
6. E. Wajnberg, M. P. Linhares, L. J. El-Jaick, and G. Bemski, *Eur. Biophys. J.* **21,** 57 (1992).
7. S. R. Kurtz and H. J. Stapleton, *Phys. Rev. B* **22,** 2195 (1980).
8. K. Lyo and R. Orbach, *Phys. Rev. B* **22,** 4223 (1980).

9. O. C. Alves and E. Wajnberg, *Int. J. Biol. Macromol.* **15,** 273 (1993).
10. H. Rupp, K. K. Rao, D. O. Hall, and R. Cammack, *Biochim. Biophys. Acta* **537,** 255 (1978).
11. E. Wajnberg, H. J. Kalinowski, G. Bemski, and J. S. Helman, *Biophys. J.* **49,** 1195 (1986).
12. M. B. Yim, L. C. Kuo, and M. W. Makinen, *J. Magn. Reson.* **46,** 247 (1982).
13. G. W. Brudvig, *Methods Enzymol.* **246,** 536, (1995).
14. C. Galli, J. B. Innes, D. J. Hirsh, and G. W. Brudvig, *J. Magn. Reson. B* **110,** 284 (1996).
15. S. K. Mun, J. C. Chang, and T. P. Das, *Proc. Natl. Acad. Sci. USA* **76,** 4842 (1979).
16. E. Wajnberg, G. Bemski, L. El-Jaick, and O. C. Alves, *Int. J. Biol. Macromol.* **18,** 231 (1996).
17. D. C. Doestchman, *Chem. Phys.* **48,** 307 (1980).
18. A. Waleh, N. Ho, L. Chantranupong, and G. H. Loew, *J. Am. Chem. Soc.* **111,** 2767 (1989).
19. J. Jortner, *J. Chem. Phys.* **64,** 4860 (1986).
20. S. Cusack and W. Doster, *Biophys. J.* **58,** 243 (1990).
21. C. P. Poole, ''*Electron Spin Resonance,*'' Wiley, New York, 1983.
22. G. E. Pake and T. L. Estle, ''*The Physical Principles of Electron Paramagnetic Resonance*'' Benjamin, Elmsford, New York, 1973.
23. V. Blombergen, S. Shapiro, P. S. Pershan, and J. Artman, *Phys. Rev.* **114,** 445 (1929).
24. K. J. Standley and R. A. Vaughan, ''*Electron Spin Relaxation Phenomena in Solids,*'' Plenum Press, New York, 1969.
25. G. Fermi, *J. Mol. Biol.* **97,** 237 (1975).
26. M. H. Rakowsky, K. M. More, A. V. Kulikov, G. R. Eaton, and S. S. Eaton, *J. Am. Chem. Soc.* **117,** 2049 (1995).
27. A. R. Drews, B. D. Thayer, H. J. Stapleton, G. C. Wagner, G. Giugliarelli, and S. Cannitraro, *Biophys. J.* **57,** 157 (1990).